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CHEMISTRY OF THE *TAXACEAE*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Chemistry of the Taxaceae" submitted by M. N. Sukumaran Nayar in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Four new compounds have been isolated by the combined use of column and thin layer chromatography from the leaves and twigs of the Western Canadian Yew, *Taxus brevifolia* Nutt.

The structures of the three major components were established and confirmed by synthesis as the following compounds, 4-(*p*-hydroxyphenyl)-butan-2-ol, 4-(*p*-benzoyloxyphenyl) - 2-benzoyloxy-butane and 4-(*p*-benzoyloxyphenyl) - butan-2-ol. A partial structure for the fourth compound, present in trace amounts only, was proposed.

Despite reports to the contrary neither "taxine" nor any other alkaloid could be detected in samples of *T. brevifolia* collected under a wide variety of ecological and seasonal conditions.

"Taxine" was readily isolated from *T. baccata*.L. In addition 4-(*p*-hydroxyphenyl)-butan-2-ol together with its mono and dibenzoyl esters were isolated.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

		Page
ABSTRACT		(i)
ACKNOWLEDGEMENTS		(ii)
PART I -	INTRODUCTION	1
PART II.	DISCUSSION OF EXPERIMENTAL WORK	
(I)	Extraction and Separation of the constituent components of *T.brevifolia* and *T.baccata*.	16
(2)	Determination of the chemical structure of the isolated compounds.	29
(3)	Conclusions.	40
PART III.	EXPERIMENTAL	
(I)	General Procedures.	42
(2)	Extraction and Separation of the components of *T.brevifolia* and *T.baccata*.	45
(3)	Chemical examination of the isolated compounds.	69
PART IV.	BIBLIOGRAPHY	87

PART I.

INTRODUCTION

The plant family Taxaceae belongs to the plant subkingdom Spermatophyta, class Gymnospermae, order Coniferales. There are ten genera and seventy-five species in the family. The genus Taxus is represented by at least six species in the north temperate zone. Over and above these, one species occurs in Mexico and another in Florida. The species indigenous to Canada are T. canadensis Marsh, also called American Yew or Ground Hemlock and the Western Yew, or T. brevifolia Nutt. The Western Yew is found on the islands and mainland of the Pacific Coast, in the southern part of the Selkirk and Rocky Mountain ranges, where it occurs as a shrub, seldom as a tree. The Western Yew is at present of no commercial importance in Canada.

The most extensively investigated species of Taxus is the European Yew, T. baccata Linn. The interest in the chemistry of Taxus arose because of the very early observation that the leaves and fruit are extremely poisonous. The first investigation was by Cornevin (1) as quoted by Whitehead (2). Also in the same journal there was a short account of experiments carried out at the Royal Veterinary College (3) on the poisonous characteristics of Yew. Reports of Yew poisoning are found in the communications of Novara (4), Aufrecht (5), Suddent (6) and Nicholson (7). However, cases have been reported where no fatal results have followed from eating Yew leaves. To explain this some have suggested that the toxicity of Yew varied with the season. Others have suggested that only leaves of the male tree are poisonous, whereas the leaves of the female tree are innocuous.

Despite the poisonous characteristics of the Yew, it has reported medicinal uses. Kondo and Ammano (8) have stated that an extract of the leaves of the Japanese Yew, T. baccata L. var. Cuspidata (Pilg) was used as an abortificant and to cure diabetes in Japan. The earliest chemical investigation of the Yew (T. baccata) was made by Lucas (9). He reported the existence in T. baccata of a specific alkaloid, to which he gave the name of taxine. In 1876 Marmé reported the properties of the alkaloid as a white crystalline substance, soluble in dilute acids, alcohol, ether, chloroform, benzene, and carbon disulphide, but insoluble in petroleum ether and water. It was odourless, had a bitter taste and gave precipitates with several alkaloidal reagents but not with platinic chloride, auric chloride, mercuric chloride and potassium platinicyanide. It melted at 80°C., turned red with concentrated sulphuric acid and gave no crystalline salts with any of the usual inorganic acids.

Amato and Capparelli (11), in 1880 attempted to isolate taxine from T. baccata. After following an elaborate extraction procedure they isolated a liquid having an odour of wild fennel, a non-nitrogenous crystalline substance m.p. 86-87°C., very difficult to crystallise and purify, to which they gave the name millossin, and an alkaloid. They have described the properties of the alkaloid as colourless, crystalline, having a musty odour, soluble in alcohol and ether and insoluble in water. It gave dense white fumes when a glass rod dipped in hydrochloric acid was held near it.

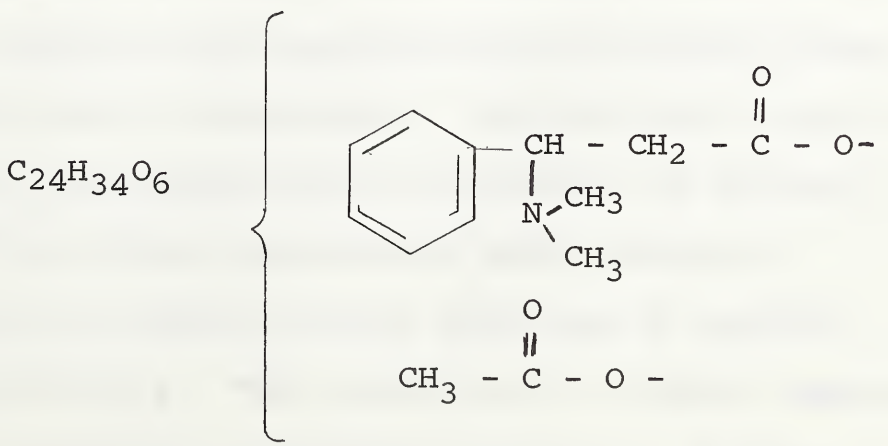
It gave a canary-yellow precipitate with phosphomolybdic acid and a white precipitate with tannin, which became crystalline on standing.

Ten years later a more detailed chemical examination of T. baccata was carried out by Hilger and Brande (12). They followed the isolation procedure of Marmé but reported the alkaloid as non-crystalline. They observed the same solubility behaviour for the substance as that of the previous workers and reported the melting point as 82°C. The evolution of a characteristic aromatic odour was noted at the melting point. The molecular formula assigned was $C_{37}H_{52}NO_{10}$. They also reported the preparation of crystalline derivatives, the hydrochloride, platinic chloride and ethiodide having molecular formulas $C_{37}H_{52}NO_{10}HCl$, $(C_{37}H_{52}NO_{10})H_2PtCl_6$ and $C_{37}H_{52}NO_{10}C_2H_5I$ respectively. From these results they concluded that taxine was a tertiary base.

In 1892 Munro (13) extracted the leaves of the male and female tree and found an alkaloid, which appeared to be taxine, which was present in slightly larger quantities in the male tree than in the female. He also pointed out the extreme difficulty of purifying taxine. Vreven (14) in 1896 also obtained taxine using a different extraction procedure. Vreven did not do any chemical examination on the isolated alkaloid.

Six years later Thorpe and Stubbs (15) carried out a detailed chemical investigation of the Yew. They reported that the most suitable method of extraction of taxine was with 1% aqueous sulphuric acid. They also recorded the general properties of the alkaloid, the molecular formula of the base, the molecular formulas of some of its salts and its colour reactions with different reagents. In addition they reported that heating of taxine with 10% aqueous hydrochloric acid for three hours decomposed 95% of the base, producing at least two substances, one of which is non-nitrogenous and amorphous, but with a melting point 120°C. The second product of decomposition was brown in colour and separated from the aqueous acid solution during heating. Ross (16) in 1909 confirmed the results of Thorpe and Stubbs.

The first significant advance in the chemistry of taxine was due to Winterstein and collaborators (17-18). They reported taxine as an amorphous substance of molecular formula $C_{37}H_{51}NO_{10}$, m.p. 105-110°C. and $[\alpha]_D$+51-52° (absolute ethanol). They prepared a methiodide $C_{37}H_{51}NO_{10}.CH_3I$, m.p. 122-125°C. The results of their investigation resulted in the proposal of the following partial formula for taxine:



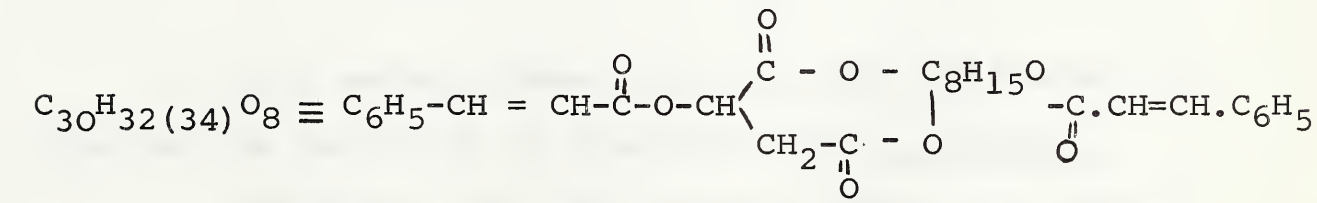
Kondo and Ammano (8) isolated taxine from the Japanese Yew, T. baccata var. Cuspidata which is botanically only slightly different from the European Yew, T. baccata. They agreed with Winterstein's results, except in optical rotation, which was reported as $[\alpha]_D$+33.34°. Also reported was the formation of a crystalline acid, m.p. 133°C., an amorphous acid, formic acid and acetic acid by the alkaline hydrolysis of taxine. They confirmed that taxine was not a glycoside. In 1925 Kondo and Takahashi (19) reported the presence of three hydroxyl groups in the taxine molecule, on the basis of the formation of a triacetyl derivative.

Hydrolysis of taxine with 0.2N alcoholic potassium hydroxide produced (1) a yellow nitrogenous substance, (2) a crystalline substance insoluble in ether, m.p. 286°C., (3) acetic acid, formic acid, citric acid and (4) a powdery acid. The nitrogenous compound had the molecular formula $C_{21}H_{31}NO_5$ with $[\alpha]_D^{21}$ - 45.51°. To this compound they gave the name taxinolamine. Decomposition of taxinolamine produced an amorphous acid, taxic acid, molecular formula $C_{16}H_{20}O_5$ and $[\alpha]_D^{20}$ - 49.29°. Taxinolamine had at least three hydroxyl groups, did not give any reaction with semicarbazone or hydroxylamine and did not form a methiodide. The two other oxygen atoms present in taxinolamine were not present as carbonyl groups. The acetyl taxinolamine with potassium permanganate in glacial acetic acid gave a compound $C_{21}H_{26}NO_6(CO.CH_3)_3$. When oxidised at a higher temperature it gave a substance $C_{20}H_{26}NO_6(O.CO.CH_3)_2$. Hoffman

degradation of taxine methiodide resulted in a nitrogen-free compound $C_{35}H_{44}O_{10}$ with $[\alpha]_D^{20}$ + 50.72°. This compound on alkaline hydrolysis gave acetic acid, citric acid, taxic acid and a neutral substance $C_{19}H_{24}O_5$, m.p. 94-95°C., which was called taxinol and corresponded to taxinolamine less $(CH_3)_2NH$. Hence, it was concluded that taxine was the ester of taxinolamine and a hydrate of taxic acid and the unsaturated alcohol taxinol produced by removing $(CH_3)_2NH$ from taxinolamine. From these results the partial structure of taxine was suggested:

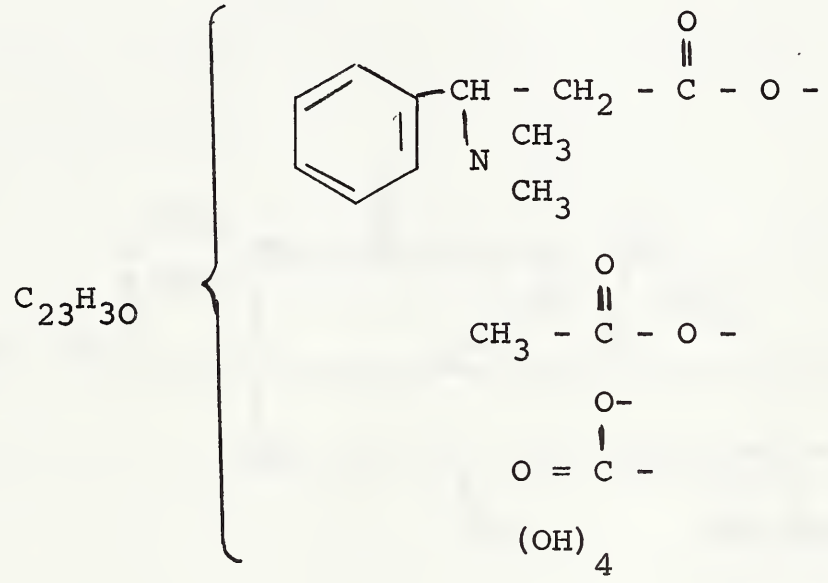
$$C_6H_5 - CH = CH \cdot C_8H_{14}O_5 \cdot C_{11}H_{14}O_2\left(N\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}\right)(OH)_3(CH_2)_2\text{-}C_6H_5$$

Six years later Thakahashi (20) reported the results of his investigation of taxinin, a nitrogen-free compound and a decomposition product of taxine. He assigned taxinin the molecular formula $C_{30}H_{32(34)}O_8$ and on the basis of the degradation products of the compound proposed the following structure for taxine:



In the same year Callow, Gulland and Virden (21) isolated taxine from T. baccata and confirmed the molecular formula as $C_{37}H_{51}NO_{10}$. They reported the isolation of β-dimethylamino-β-phenyl propionic acid from taxine after hydrolysis with boiling dilute sulphuric acid. The other product of hydrolysis they called anhydroxatine $(C_{24}H_{36(34)}O_7)$.

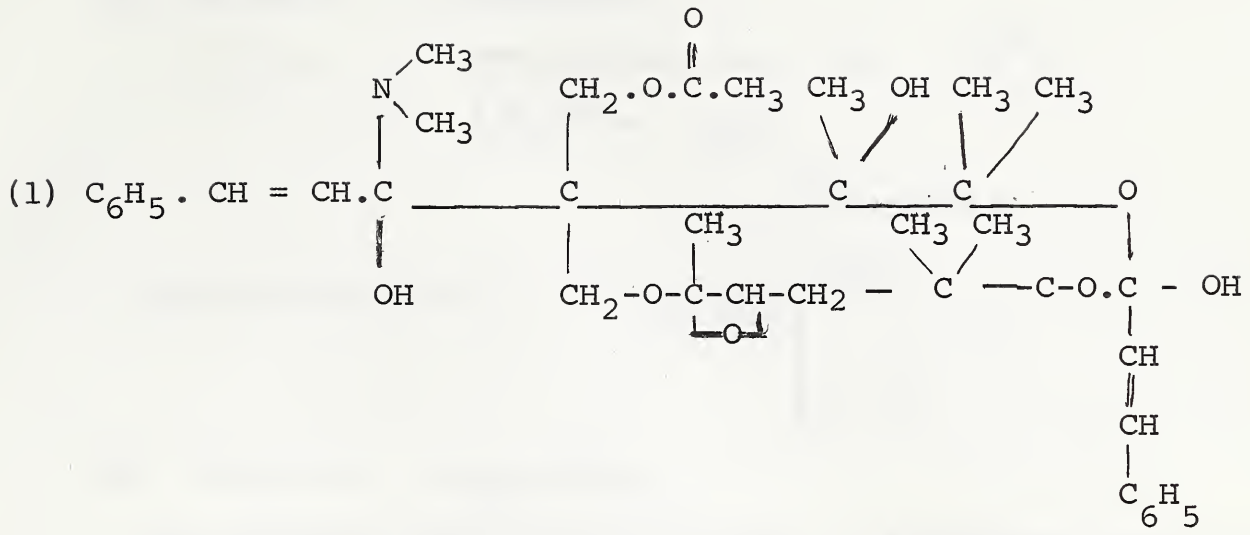
Anhydroxatine contained no methoxyl group, no ketonic or aldehydic carbonyl group, and was not oxidised by Fehling's solution. Nevertheless it reduced Tollen's reagent. Further, it was unsaturated towards permanganate and behaved as a lactone towards alkali. On the basis of these observations they concluded that anhydroxatine was an unsaturated lactone of the type of angelicolactone. They had accounted for all the oxygen atoms and also the nitrogen atom and proposed the partial structure of taxine as:



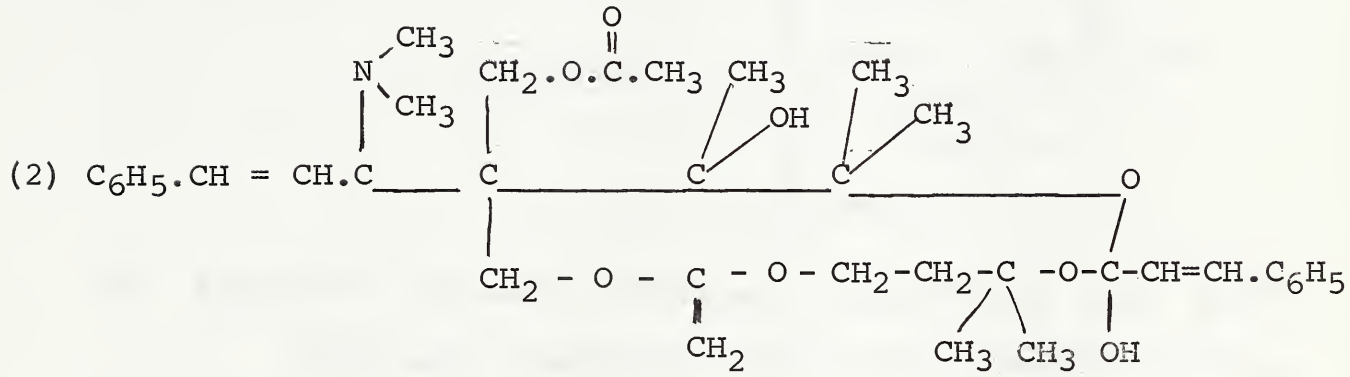
In a second communcation Gulland and Virden (22) reported the isolation of ephedrine from T. baccata.

Takahashi (23) made further studies on taxinin. He revised some of his previous results and also gave the results of hydrogenation, oxidation and hydrolysis reactions. The same author (24) in 1934 further revised the results of his previous work and finally proposed two alternate structures for taxine:

(1)



(2)



Twenty-two years later Graf (25) described in a lecture his results on the chemical investigation of taxine. Here he reported that taxine was a mixture of alkaloids, which he called taxine A, taxine B, and taxine C. The latter was present only in very small amounts. He also proposed a partial structure for taxine A and taxine B:

(1) taxine A - $C_{35}H_{49}NO_{10}$

crystalline, m.p. 204 - 206°C.

$[\alpha]_D^{20} = -140^\circ$

partial formula -- $C_{27}H_{38}N\{(CH_3.COO)_3, (OH)_2, -C=O, C-O$

(2) taxine B - $C_{35}H_{51}NO_{10}$

Approximately 30% of the mixture of alkaloids, m.p. 111 - 113°C.

$[\alpha]_D^{20} = +119^\circ$.

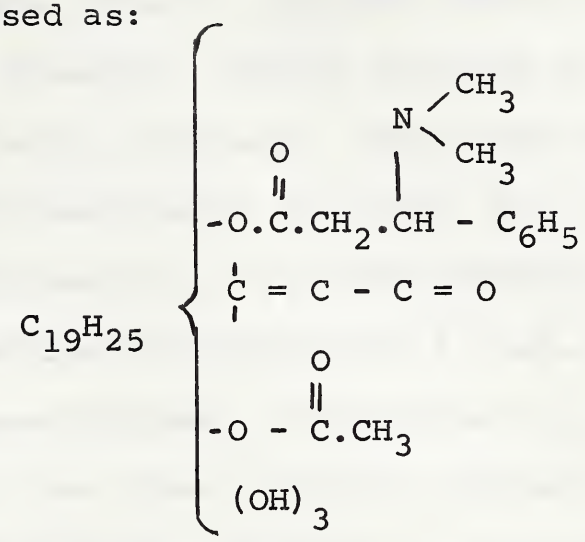
Amorphous - forms a crystallizable diacetate.

partial structure -- $C_{22}H_{29}O_3\{CH_3COO-, C_6H_5CH(N(CH_3)_2)-CH_2.C(=O)-O-, (OH)_3$

(3) taxine C - present in traces, crystalline, m.p. 211°C.

Graf and co-workers (26-29), in a series of four papers, elaborated the results of their previous communication on taxine A and also revised the molecular formula of taxine B to $C_{33}H_{45}O_8N$ and also on the basis of ultraviolet and infrared spectra proposed the presence of an $\alpha\beta$ unsaturated carbonyl group. They also confirmed the presence of β-dimethylamino β-phenyl propionyl grouping in the molecule by acid hydrolysis. They also investigated the optical activity and configuration of β-amino β- phenyl propionic acid and its N-methyl

derivatives. The amended partial formula for taxine B was proposed as:

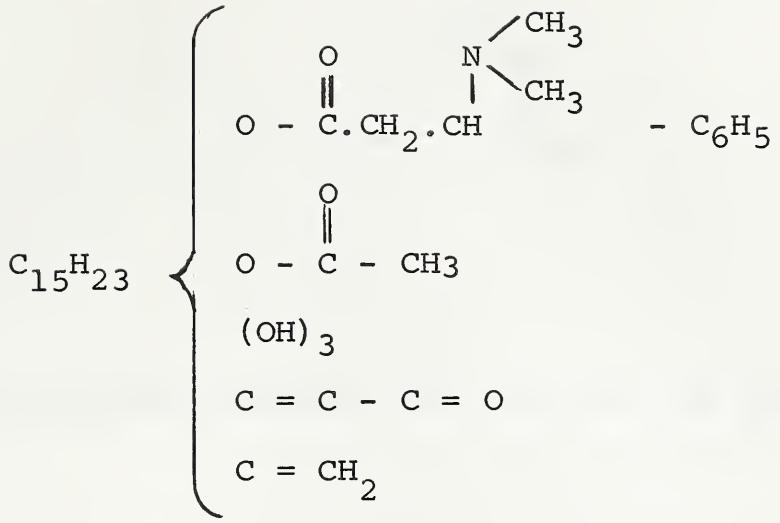


It was also speculated that the main carbon skeleton of the alkaloid may be diterpenoidal.

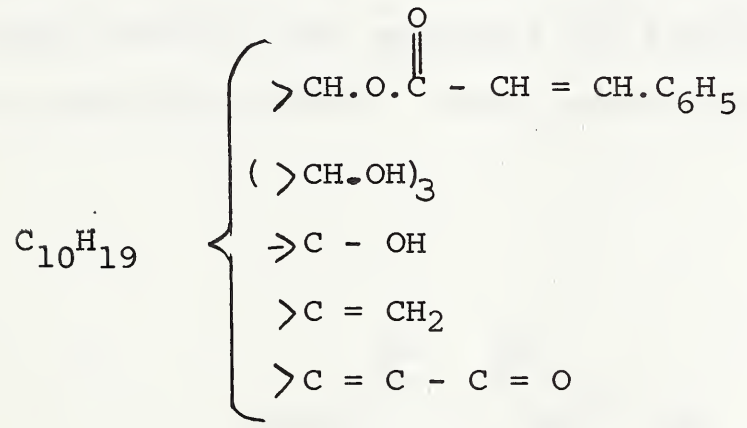
The Japanese workers Kondo, Taga and Takahashi and their co-workers continued their investigation of taxinin and published their findings in a series of papers (30-34). They further revised the molecular formula of taxinin from $C_{30}H_{32(34)}O_8$ to $C_{31}H_{38(40)}O_8$. They also discussed the results of catalytic hydrogenation, selenium dehydrogenation, lithium aluminum hydride reduction and hydrolysis reactions and also the ultra-violet and infrared spectra. Their work regarding taxinin is contradictory and confusing.

Baxter, Lythgoe, Seales and Trippett (35) published the results of their investigation of T. baccata L. They have also shown that taxine is a mixture of alkaloids and have named the major alkaloid of this mixture as taxine I, molecular formula $C_{33}H_{45}O_8N$. By treating the crude taxine methiodide with cold potassium carbonate solution in water they eliminated trimethylamine from

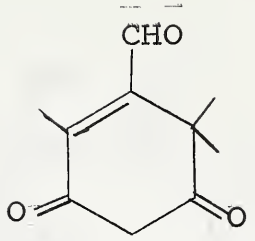
the "taxine" mixture of alkaloids, producing the mixture of cinnamate esters. Zemplén removal of the acetyl groups from the cinnamate complex followed by crystallisation gave the major component, which they have named as O-cinnamoyltaxicine-I, $C_{29}H_{36}O_7$, m.p. 224°C. $[\alpha]_D$ + 279°. O-cinnamoyltaxicine-I by hydrogenation was converted to O.β. phenylpropionyltaxicine I, m.p. 207°C. $[\alpha]_D$ + 176°. This compound formed a triacetate m.p. 179°C. $[\alpha]_D$ + 138°, containing one free alcoholic hydroxyl group. They have suggested that the alkaloidal precursor of these compounds occurs mainly as the monoacetate of O-β-dimethyl amino-β-phenylpropionyltaxicine-I $C_{33}H_{45}O_8N$. It was also suggested that the parent compound taxicine-I, $C_{20}H_{30}O_6$ was diterpenoidal. It decomposed in alkaline solution. In their further studies (35) they have chosen O-cinnamoyl-taxicine-I as the starting material. This compound was shown to contain four hydroxyl groups, some of which are vicinal, as indicated by periodate oxidation studies. O-β-phenylpropionyltaxicine-I, a controlled hydrogenation product of O-cinnamoyltaxicine-I, was shown to contain an isolated methylene double bond. This was shown by oxidising the triacetate of O-β-phenylpropionyltaxicine-I with potassium permanganate producing a glycol, which was split be lead tetraacetate to formaldehyde and a carbonyl compound $C_{34}H_{42}O_{11}$, which formed an oxime. On the basis of these results and spectral evidence they proposed a partial formula for taxine-I:



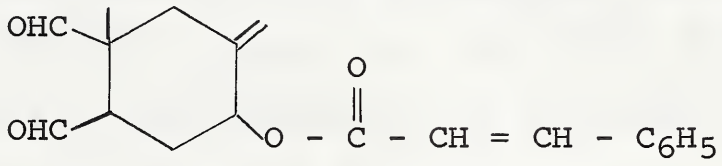
More recently Baxter, Lythgoe, Scrowston and Trippett published (36, 37) further results of their investigation of taxine-I. In their investigation they have chosen O-cinnamoyltaxicine-I as their starting material. They have established the functional groups in this molecule on the basis of chemical and physical evidence as:



By a periodate cleavage of O-cinnamoyltaxicine-I these workers obtained two fragments, an acidic and a neutral one, which together accounted for all the carbon atoms in the molecule. By degradation experiments and also on the basis of spectral evidences they have shown that the acidic fragment had the structure:

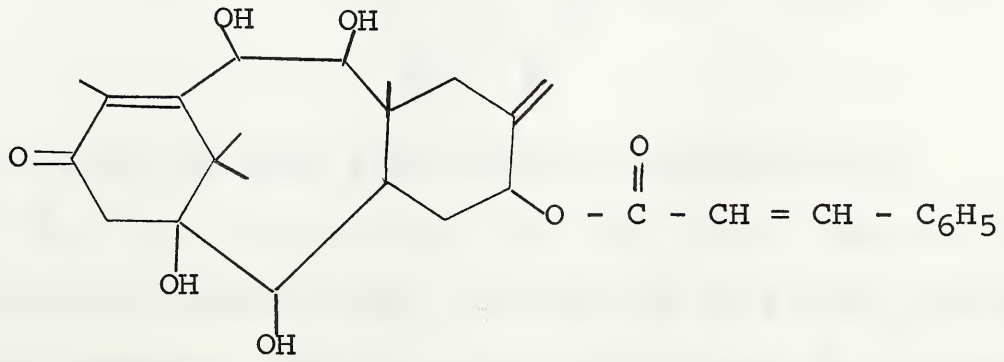


The neutral fragment was assigned the structure:

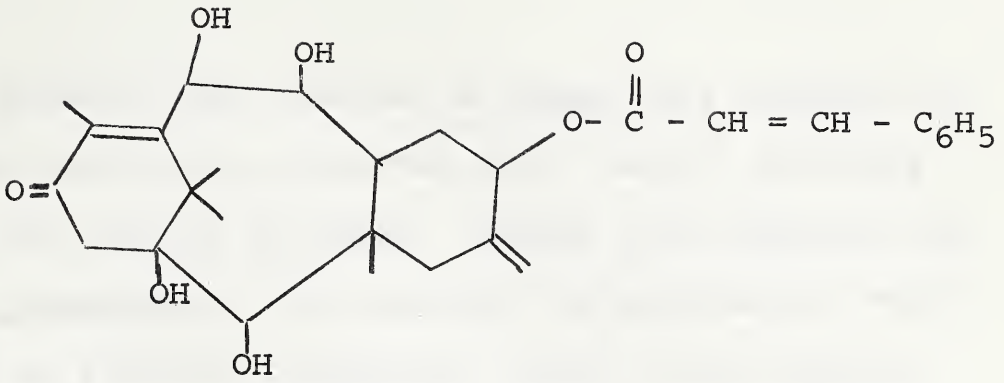


The structure of the neutral fragment was confirmed by degradation studies and spectral evidence. On the basis of these results they proposed two alternate structures for O-cinnamoyltaxicine-I. These structures were:

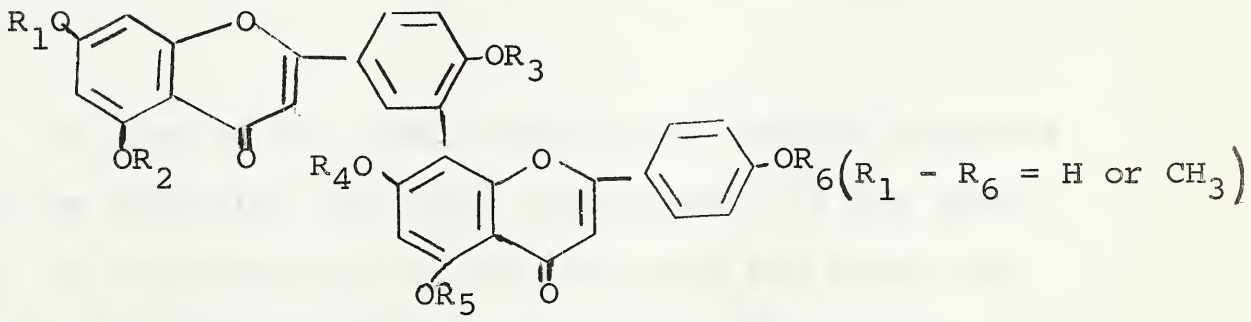
1.



2.



Other than the alkaloidal mixture "taxine" there have also been isolated from T. baccata a glucoside called taxicatin (38,39), $C_{14}H_{20}O_8$ m.p. 169-170°C. $[\alpha]_D^{20}$ - 67.25°. The structure of this glucoside was confirmed by synthesis (40). Over and above this glucoside several common sugars and a rare sugar 2-O-methyl L-fucose (41), which was previously unreported in nature, were isolated. Three biflavanoidal pigments have also been isolated from T. baccata (42-44) which have been shown to have the general structure:



One of them has been identified as sciadopitysin ($R_1 = R_2 = R_6 = CH_3$ and $R_2 = R_4 = R_5 = H$). The two other biflavanoid pigments were isolated in very small amounts from T. baccata. One of them is an isomer of sciadopitysin differing only in the position of the methoxy groups.

Several other species of Taxus were examined by Hegnaur (45) and he has reported that "taxine" probably occurs in all species of Taxus. Masson (46) examined the species T. canadensis and reported the presence of small amounts of an alkaloid resembling taxine in its general reactions. He also reported the presence of a hitherto unreported glycoside, molecular formula $C_{16}H_{26}O_{11}$, the structure of which has not yet been established. Bourbeau (47) has reported the isolation of an entirely new alkaloid $C_{37}H_{57}N_3O_{10}$, which he has named taxinin.

T. brevifolia Nutt was first investigated by Johns and Lynn (48). They found no volatile oil, no alkaloid and no glycoside in this species. Massor (49) has reported the presence of "taxicatin" probable, whereas Tyler (50) has reported the presence of small amounts of taxine in this species.

In view of the wide diversity of results reported both for the alkaloids and other constituents of the genus Taxaceae, an investigation of the alkaloids and other constituents of T. brevifolia and subsequently T. baccata was undertaken.

PART II

SECTION (i)

DISCUSSION OF EXPERIMENTAL WORK

(1) Extraction and Separation of the constituent components of T. brevifolia and T. baccata.

Despite the fact that chemical investigations on constituents of Taxaceae have taken place for over a century, the information available from this mass of research is inconsistent, contradictory, confusing and in many cases chemically unacceptable. An example of this chaotic state can be seen in the results reported by the Japanese group of workers (8, 19, 23, 24, 30, 31, 32, 33, 34) who have published the largest number of papers on T. baccata L. var. cuspidata. They have revised the results in their previous communication in almost every subsequent publication but the end result has in no substantial way contributed to the solution of the problem. Others have reported (21) that they were unable to reproduce some of their own reported results. The most widely examined species of the Taxaceae is T. baccata Linn. All those who investigated this species prior to Graf (25) have stated that it contains an alkaloid "taxine" and have reported reactions and properties of this alkaloid. These are for the most part contradictory. Graf (25) in his first communication pointed out that the so-called "taxine" was a mixture of three alkaloids. This was confirmed later by Baxter and his co-workers (35).

On one point, however, most of the investigators are in agreement, in that "taxine" is extremely unstable both in solution and in the solid state (15, 21). Two other important observations were that "taxine" decomposes in aqueous

hydrochloric acid and a stable "taxine" derivative (dihydro-O-β-phenylpropionyltaxicine-I) rearranges under mild conditions in aqueous hydrochloric acid (35).

One of the less widely investigated species of Taxus is T. brevifolia, or the Western Yew. With this species, as with T. baccata, conflicting and contradictory reports concerning the chemical constitutents have been published. One group claimed the complete absence of alkaloids, heterosides and volatile oils (48), whilst a second claimed to have isolated "taxine" (50).

At the outset of the present investigation it was patently obvious that the key to the successful structural elucidation of the components of the Taxus species was the extraction of the plant components under the mildest conditions, followed by the separation of the mixture so obtained into single components. These procedures were in turn dependent upon the establishment of a rapid physical method with which to follow these separation techniques.

The gross extraction procedures used were cold percolation and soxhlet extraction. In both cases the finely powdered plant material was pre-extracted with n-heptane to remove lipids and part of the colouring materials. Two separate solvents were used in the cold percolation technique; (a) 5% V/V acetic acid in methanol and (b) 2% V/V aqueous sulphuric acid. Methanol

was used in the soxhlet extraction method. In all cases the plant material was extracted until evaporation of a portion of the extract left little or no residue. The methanol extracts were concentrated by vacuum distillation to a small volume before proceeding, whereas the aqueous acid extract was used as such. In the case of methanolic extracts, precipitation of water insoluble materials, mainly chlorophyll, was followed by filtration. This gave an amber coloured filtrate which was then extracted with diethyl ether at various pH values. The ether extract gave, after evaporation 'in vacuo' a pale yellow viscous liquid, the E series extracts. In the case of the acid percolate, the percolation was stopped when the extract failed to give positive Mayer's test. The percolate was made basic and extracted with ether; this ether extract on evaporation gave a pale yellow viscous oil. The only gross difference in the weights of the extracts obtained was that of the soxhlet extraction, which gave almost twice the yield of extracts. The soxhlet method because of its efficiency and ease was used for all further extractions of plant material.

Both the acid (E 7) and basic extracts (E 5; E 6, and E 8) from T. baccata gave a positive Mayer's test (alkaloid), as did the acid extract (E 3) from T.brevifolia. There was little if any basic extract

(E 4) obtained from T. brevifolia. Although samples of T. brevifolia were obtained from trees having different locations and also from the same location at different times of the year little or no basic material (E 4 a, E 4 b, E 4 c) could be obtained from any of these plant materials. This was in agreement with the findings of Lynn and Jones (48) but diametrically opposed to the more recent work of Tyler (50). The later work is however open to severe criticism, since the isolation and purification techniques used to obtain 'taxine' would have completely decomposed the "taxine" alkaloidal complex.

It was expected that paper chromatography of the extracts would reveal the number of components and could also be used to follow the column chromatographic separation of these extracts. However, whilst a new spray reagent, 1% aqueous solution of potassium ferriccyanide, followed after drying with 1% aqueous ferric chloride proved to be very sensitive, no separation of the components present in a typical extract E 3 could be achieved. Since no separation was found, further work on paper chromatography was abandoned. It was hoped that the individual components of the extracts could be separated by column chromatography or by similar mild methods.

It was not until sometime later that the then little used Thin Layer Chromatography (TLC) was investigated for use as a rapid physical method of separation and detection. TLC proved to be an extremely efficient and rapid method not only for qualitative separation of the components of the extract but also as a means of following the separations achieved by adsorption column chromatography and it was therefore not until much later that TLC was used to follow the column separations. It was found that a 0.25 mm thick layer of silica gel G (Merck) on a glass plate, when developed under the supersaturated chamber technique (55) with either a methanol/chloroform/n-hexane/diethylamine (3:10:20:2.3) or a methanol/chloroform/n-hexane (11:20:40) solvent mixture gave an excellent separation of the individual components. The acid extract E 3 was found to contain four main components which are referred to hereafter as compounds A, B, C, and D together with a minor component E. These had R_f values 0.38, 0.41, 0.58, 0.66, and 0.96 for an 8 to 9 cm. solvent run. The materials had no ultraviolet fluorescence, but were easily detected by a ceric sulphate (1%)/10% aqueous sulphuric acid spray (51). This spray reagent gave a violet colour with compound A at room temperature and dark brown spots with all other compounds after heating the sprayed plate at 110°C

for 3 - 5 minutes. The extreme sensitivity of the reagent when used with almost any organic material makes this mixture an almost ideal spray reagent for TLC, when inorganic materials are used to make the thin film.

It was now possible by the use of TLC to evaluate in more detail the different extraction procedures that had been used (see part III section (i)). All methanol based extracts of T. brevifolia were found to contain compounds A, B, C, D, and traces of E, and in addition some material $R_f < 0.3$ could be detected but was not as sharply separated as the rest of the components. A, was in all cases the major component of the extracts. The heptane extract showed chlorophyll and related pigments near the solvent front but only trace amounts of A, B, C, D, and E were found. Extract E 2 contained the major component A and was otherwise similar in all respects to those already described.

The extracts derived from T. baccata showed significant differences in the basic extracts (E 5, E 6, and E 8). These should correspond to the "taxine" alkaloidal complex. TLC of these extracts showed three distinctly coloured spots when sprayed with the ceric-sulphate spray reagent, R_f 0.81, 0.88, and 0.92, without heating. On heating, there was also present, a series of closely separated spots R_f 0.15 - 0.30, in addition to the previously described spots.

In contrast extract E 7 was qualitatively identical to similar extract E 3 from T. brevifolia. It contained A, C, and D. TLC separation patterns were virtually identical for all the three different primary extraction procedures. The soxhlet extract with methanol did however show a somewhat higher concentration of A.

In view of the more ready availability of T. brevifolia and also that it appeared to contain less components than T. baccata, work was initially undertaken on the former species.

In an effort to effect a preliminary separation of the components of the extract E 3, three methods were used, an acid/base/neutral separation, short path vacuum distillation and steam distillation.

The acid/base/neutral separation failed to effect any significant fractionation and was accompanied by a large initially unaccountable overall loss of material, which was found later to be due to the relatively high solubilities of the components in water. Whilst three fractions were obtained TLC showed these to be essentially the same. Infrared spectra of these fractions were also virtually identical.

Short path vacuum distillation at 40°C of the extract E 3 was now tried when two main

distillable fractions and a deep brown non-volatile residue were obtained. One of the volatile fractions was a colourless crystalline compound m.p. 120.5-121°C. which was readily identified as benzoic acid. The second, a pale viscous oil was found by TLC to be almost pure A with only slight traces of D present. The residue contained only A together with other new unidentified components ($R_f < 0.30$) and also deep brown colouring material. Since the yields of benzoic acid and A were low this method was not further used for the isolation of A or benzoic acid.

Steam distillation of extract E 3 was more successful. An ether extract of the steam distillate gave on evaporation a small amount of a volatile liquid which had a peculiar odour. TLC showed this to be E containing trace amounts of A. The golden brown supernatent liquor left in the flask after steam distillation was decanted from a brown resinous mass. The aqueous liquor when extracted with ether gave after evaporation a light brown viscous oil, shown by TLC to be mainly A with traces of compounds $R_f < 0.3$. The brown resin was soluble in chloroform which gave after evaporation a dry viscous residue. This residue was extracted first with cold, then hot benzene. The hot benzene extract after evaporation gave a pale yellow

viscous oil composed mainly of D with traces of C. The dark brown residue obtained by evaporation of the cold benzene extract consisted mainly of A with some B, and C and traces of D.

In an attempt to obtain pure D from the mixture of D and C (obtained from the hot benzene extract), separation by column chromatography on alumina (activity $\overline{V}$) was employed. Only relatively small amounts of compound D were recovered from the column. Instead, A and C were obtained. An infrared spectrum of D showed an absorption band at 1779 $cm.^{-1}$. This was not found in the infrared spectrum of compound A isolated from a column separation. Alkaline hydrolysis of D obtained from the above column ($\overline{\overline{VII}}$ fraction 5), gave, after working up, benzoic acid and A. Therefore D can be considered as a benzoylated A.

The cold benzene extract from the steam distillation residue contained a higher concentration of B relative to the starting material (E.3), of the experiment. The separation of B from this enriched fraction was possible by alumina column chromatography. But complete separation of B from A and C was only possible after repeated chromatography on alumina followed by a silica gel column separation.

Meanwhile, previous to the above three preliminary gross component separations, column chromatographic fractionation using alumina, activity $\overline{V}$ had given three major eluate fractions and had different infrared spectra. The spectral differences were mainly in the carbonyl region (1780 - 1690 $cm.^{-1}$). It was only by repeated column chromatography that these three distinct fractions were obtained. With the development of the TLC technique the identification of the components of the eluate fractions became a simple routine matter. The main fractions previously isolated after lengthy procedures were now found to contain mainly compound A but with differing amounts of B, C, and D. It was at this stage possible to select the steam distillation method for the gross separation of the components of the extract E 3 of T. brevifolia, to be supplimented wherever necessary by column chromatography.

Initially column chromatography had been the only method available by which a separation of the components of the plant extracts could be satisfactorily followed. Even so, this was dependent on slight differences in infrared spectra and on differences in physical states of the eluate fractions. It was not until after the successful development of TLC that the separation observed on adsorption columns could be rationalised. At that time it was obvious that to attempt a

separation of the total extracts by column chromatography was inferior to the steam distillation technique. Nevertheless the knowledge obtained from the multitude of column separations was invaluable for the purification of the enriched fractions, obtained by steam distillation and the other methods.

Three major fractions were obtained from the crude extract when it was separated on an alumina column (column II). These were the benzene, anhydrous chloroform, and 1.5% v/v methanol/chloroform eluates. The benzene eluates gave a volatile liquid whose boiling point was close to that of benzene. TLC showed this to be E. Several individual fractions eluted with anhydrous chloroform, gave a viscous yellow liquid which after standing for 8 - 12 weeks at $0^{o}C$ partially solidified. TLC showed this to be A with traces of B. The 1.5% methanol/chloroform eluates also gave a material solid at room temperatures, but non-crystalline. The infrared spectrum of both the eluates showed strong hydroxyl absorption (3600 - 3300 $cm.^{-1}$ envelope). TLC showed this also to be mainly A with traces of B.

In an attempt to improve the separation a portion of eluate fractions (131 - 140), from alumina column II, was acetylated and chromatographed on

an alumina column. The first two fractions eluted with benzene constituted over 50% of the total material; however, late benzene eluates had some starting material.

At this point TLC was developed and used for the first time to aid the separation of the Taxus species constituents. The eluate fractions obtained by column chromatography were found to be mixtures. Fractionation of these mixtures was not possible by column chromatography.

Since TLC proved to be extremely efficient in the qualitative detection of the components and effected good separation, it was decided to use TLC in a preparative manner. The thickness of the silica gel layer was increased to 1 mm. and the sample applied in a continuous line instead of spaced spots. Detection of the chromatograms in the standard way, was by the elegant water spray method of Bishop and Tate (52) which gave opaque bands on a translucent background. The opaque zones were marked and removed after drying by the "micro vacuum cleaner" technique. Elution of the silica gel either in the cold or by a microsoxhlet gave TLC pure compound A. This was found to be a colourless oil, which would not crystallise from solvents but slowly solidified to a crystalline mass at -70°C. Eluate fractions (131 - 140) from column II

composed of A and traces of B was acetylated and purified by alumina column chromatography which gave a single component.

When micro-analytical data for A and acetylated A were received it was found that A contained nitrogen whereas acetylated A did not. Saponification of the acetylated material gave only A. Furthermore, compound A before TLC purification did not give a qualitative test for nitrogen. When the alternate solvent system, which did not contain diethylamine, was used analytical results showed the absence of nitrogen. The anomalous analytical results can be explained by the non-stoichiometric retention of the diethylamine by the glass like material. It was extremely difficult to obtain compound A and in general all the compounds encountered in this investigation, free from solvents. Only one compound in the whole investigation was obtained in a solid crystalline state.

DISCUSSION OF EXPERIMENTAL WORK

SECTION (ii)

DETERMINATION OF THE CHEMICAL STRUCTURE OF THE ISOLATED COMPOUNDS

Since compound A was by TLC the major component of all extracts of T. brevifolia and closely related if not identical to F, of T. baccata and also had been isolated in highest yield it was the first compound to be systematically investigated.

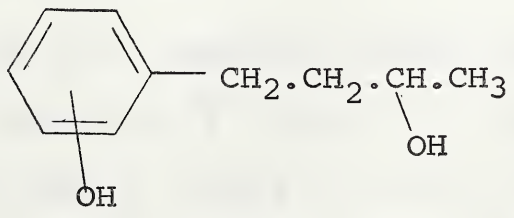
Initially it was assigned the empirical formula $C_{37}H_{51}NO_{10}$, on the basis of microanalytical data obtained from a sample purified by preparative TLC, using a solvent containing diethylamine. The nitrogen analysis was later shown to be due to a non-stoichiometric retention of diethylamine by the viscous compound A. The assignment of nitrogen was not initially questioned since the compound gave all the qualitative precipitation test for organic bases. Further evidence for the initial empirical formula was that A rapidly reacted with lead tetraacetate to consume 5 moles of the oxidant which would correspond to the cleavage of vicinal hydroxyl groups and the acetylated A did not consume any lead tetraacetate.

However, it was difficult to reconcile the $C_{37}H_{51}NO_{10}$ with other data available. The acetylated A did not contain nitrogen. Its molecular weight both by the Rast and vapour pressure methods was 258 - 260. The infrared spectrum was not consistant with vicinal hydroxyl groups. It was at this point after weighing all the contradictory evidence which will not

be discussed that it was found that in fact the nitrogen was due to trace contamination.

The alteration of the TLC procedure finally led to the isolation of A as a nitrogen free colourless viscous oil. This had a molecular formula $C_{10}H_{14}O_2$ which on acetylation by the pyridine/acetic anhydride method gave another colourless viscous oil of molecular formula $C_{14}H_{18}O_4$. The latter corresponded to diacetyl A. Alumina column chromatography of the acetylation products of a sample of A containing traces of B, easily separated diacetyl A from acetylated B. Diacetyl A, under mild conditions, was readily deacetylated to give A, thus proving no structural changes occurred under the acetylation conditions used.

It was an examination of the physical data of A and diacetyl A together with several qualitative tests that suggested the following tentative structure for A to be proposed.



Infrared spectrum of A (as a thin film) showed a broad absorption envelope at 3275 cm.$^{-1}$, assigned to intermolecular bonded hydroxyl groups. The lack of significant absorption between 1800 - 1600 cm.$^{-1}$ excluded a normal carbonyl group. The carbonyl group could not be excluded entirely on spectroscopic evidence since some hydroxy ketones fail to show typical ketone absorption. But the formation of a diacetyl derivative and its infrared spectrum almost completely excluded a carbonyl group. Absorption at 1600 cm.$^{-1}$ and 1510 cm.$^{-1}$ was typical of c = c - aromatic skeletal in plane vibration. A series of absorption bands from 840 - 690 cm.$^{-1}$ were characteristic of a disubstituted aromatic system and in addition a strong band at 824 cm.$^{-1}$ was suggestive evidence of a para-disubstituted aromatic system. However, since it is known that polar substituents on aromatic systems cause both intensification and shifts of both the in plane and out of plane banding vibrations of aromatic hydrogens a definite assignment was not made.

The U.V. spectrum showed intense end absorption with λmax 2033 Å (log ϵ = 3.74); 2247 Å (log ϵ = 3.87) and 2800 Å (log ϵ = 3.77). Addition of base gave a marked bathochromic shifts λ max 2119 Å (log ϵ = 3.82) 2427 Å (log ϵ = 3.89) and 2966 Å (log ϵ = 3.79). The bathochromic shift from 2800 - 2966 Å

(166 Å) coupled with intense end absorption maxima is characteristic of a monophenol. Compound A gave a positive iodoform reaction and coupled with benzene diazonium chloride to give an orange red precipitate of an azo dye. The N.M.R. spectrum gave a series of signals 3 - 3.40 τ corresponding to four aromatic protons; in addition the (split peak) three proton signal at 8.78 τ corresponded to a secondary C-methyl group with an electronegative substituent on the adjacent carbon atom. Two, two proton signals at 7.37 τ and 8.27 τ were consistent with a $-CH_2-CH_2-$ assignment. Whilst a one proton signal at 6.19 τ was constant with the structure $-\underset{|}{C}\underset{|}{H}$ OH.

All the spectral data on A are consistent with the suggested structure on page 30. Additional evidence was forthcoming from the I.R. U.V. and N.M.R. spectra of diacetyl A. The I.R. showed no absorption from 3700 - 3200 $cm.^{-1}$ indicating the complete esterification of the hydroxyl groups of A. Absorption of 1765 $cm.^{-1}$ and 1735 $cm.^{-1}$ were characteristic of acetyl -O- aryl and acetyl -O- alkyl groups respectively; in addition the aromatic c=c absorption 1600 $cm.^{-1}$ (weak) and 1510 $cm.^{-1}$ was still present. The U.V. spectrum showed slight differences from A. After the addition of base, a rapid scan did not reveal a bathochromic shift. However, after standing for a few minutes a shift became apparent due to ester hydrolysis. N.M.R. spectra confirmed the diacetyl analytical figure; since two new

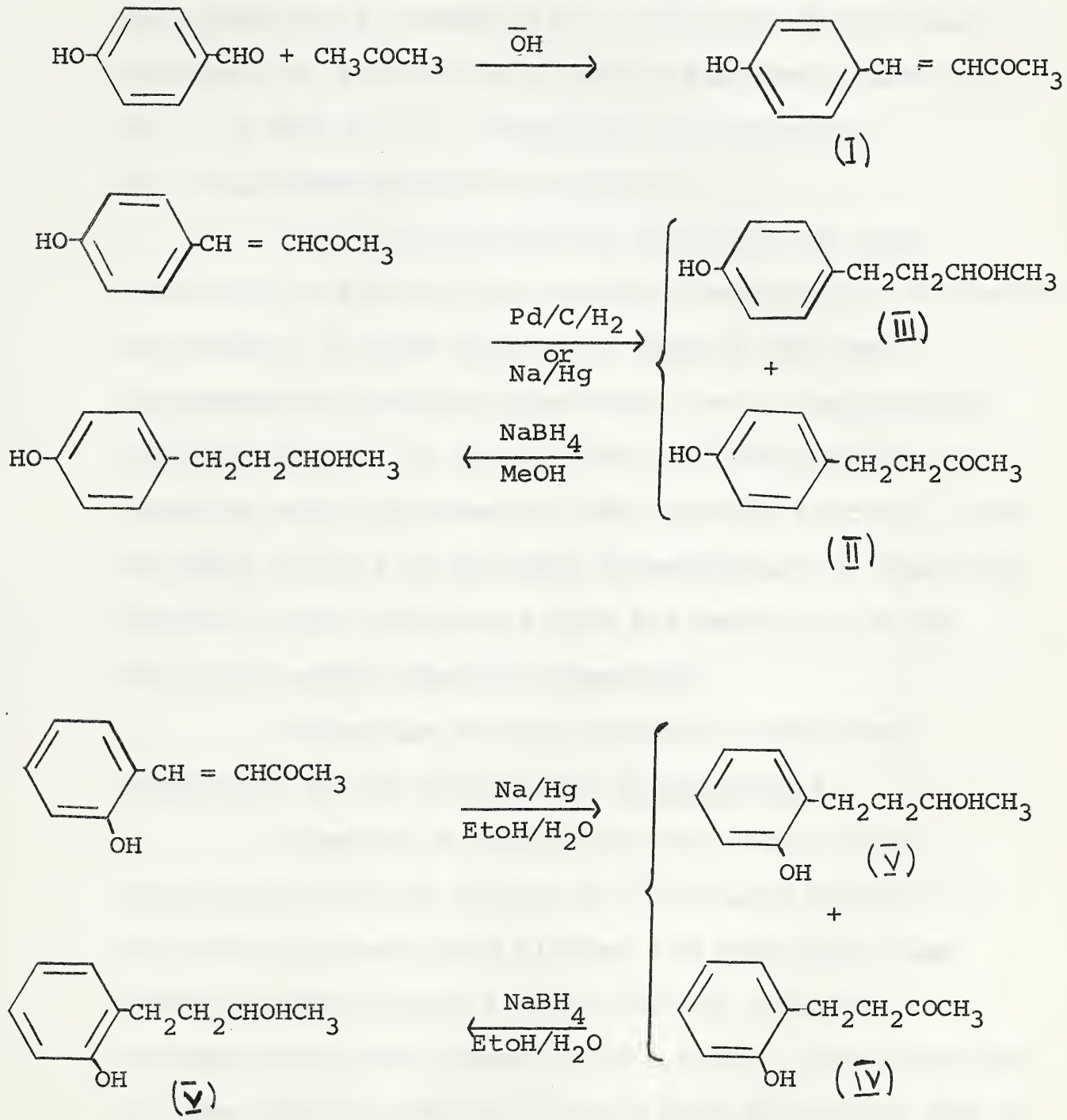
three proton signals at 7.75 τ and 7.95 τ were found. The absence of the one proton signal at 4.4 τ and the appearance of a one proton signal at 5.4 τ suggest the grouping $-\underset{|}{C}\underline{H}.OCOCH_3$.

It was now obvious that A had the gross structure previously proposed but the final aromatic substitution pattern would have to be settled by synthesis of the three possible isomers. It was however felt that a 1:3 disubstitution was unlikely. The 1:2 and 1:4 substituted compound were therefore synthesised. A Schmidt-Claisen condensation of p-hydroxybenzalde with acetone under basic conditions (59) gave 4-(p-hydroxyphenyl) -3-buten-2 one (I). This was obtained as pale yellow crystals m.p. 110.5 - 111°C. Zèmplen (59) reported 112 - 113°C. and Mannich (60) as 112°C. The I.R. absorption at 1670 $cm.^{-1}$ (-CO-CH=CH-Ar) and U.V. absorption at 3270 Å (log = 4.26) are in agreement with the expected structure (I).

Catalytic hydrogenation of (I) using palladium/charcoal as catalyst was reported by Zèmplen to give 4-(p-hydroxyphenyl)-butan-2-one (II). Hydrogenation of (I) in ethanolic solution with palladium/charcoal as catalyst failed to give this selective hydrogenation, but, instead gave a mixture of (II) and 4-(p-hydroxyphenyl)-butan-2-ol (III). This was clearly shown by TLC when (II) and (III) were sharply separated; R_f 0.43 and 0.38 respectively. Since (III) alone was required, the mixture from the hydrogenation was treated with sodium borohydride to give (III) as the lone reaction product. (III) was found

to be identical in all respects to compound A. Like compound A the synthetic product (III) was a viscous oil, which solidified to a crystalline mass on prolonged cooling. The fine needles so obtained had a melting point of 67 - 68°C. Previous melting points reported are 70 - 71°C. (59), 69 - 70°C. (60), 68 - 70°C (61) and 81°C. (62). Compound A and (III) had no observable optical rotation from 3000 Å to 6000 Å. However 4-(p-methoxyphenyl)-butan-2-ol has been resolved by Delepine and Sosa (64) to give the optically active isomers: disomer, $[\alpha]_{436}^{14}$ + 36.5°, l-isomer, $[\alpha]_{436}^{14}$ - 37.5°. (III) and compound A are therefore the dl mixtures.

It seemed of interest to attempt to selectively reduce the carbon-carbon double bond in (I) to prove or disprove whether or not Zemplen (59) had in fact obtained (II) by hydrogenation of (I). A solution of (I) in 95% ethanol was shaken with 2% sodium amalgam when it was hoped that either all (II) or possibly only (III) could be obtained. After isolation of the reaction product it was found that both (II) and (III) were formed. This product was then reduced as before with sodium borohydride to give (III). The reduction of (I) to (II) and (III) by this method gave essentially the same product as catalytic hydrogenation. The method was therefore used in the synthesis of 4-(o-hydroxyphenyl)-butan-2-ol. 4-(o-hydroxyphenyl)-buten-2-one was reacted in 95% ethanol solution with 2% sodium amalgam under the conditions previously described. The crude reaction


HO
CHO + CH3COCH3
OH
HO
CH = CHCOCH3
(I)
HO
CH = CHCOCH3
Pd/C/H2
or
Na/Hg
HO
CH2CH2CHOHCH3
(III)
+
HO
CH2CH2COCH3
NaBH4
MeOH
HO
CH2CH2CHOHCH3
(II)
CH = CHCOCH3
OH
Na/Hg
EtoH/H2O
CH2CH2CHOHCH3
OH
(V)
+
CH2CH2COCH3
OH
(IV)
NaBH4
EtoH/H2O
CH2CH2CHOHCH3
OH
(V)

product was treated with sodium borohydride to give 4-(o-hydroxyphenyl)-butan-2-ol (V). This compound was not identical to compound A. It did not crystallise and the I.R. spectrum was totally different, (from 1500 $cm.^{-1}$ to 600 $cm.^{-1}$). Compound A is therefore dl- 4-(p-hydroxyphenyl)-butan-2-ol.

It is of interest at this point to draw attention to some of the anomalous reactions of A already mentioned. It gave a positive Mayer's test and Dragendorff's reaction presumably due to the phenolic hydroxyl group. It reacts with lead tetraacetate, the reaction again dependant on the phenolic function. The variable results of the Rast determination of molecular weight is also consistent with the behaviour of low molecular weight phenolic compounds.

Attention was now focused on the other components of the extracts of T. brevifolia.

Compound D, which was obtained by steam distillation of the extract E 3 contained traces of C. As previously mentioned alumina (activity V) column chromatography caused a large loss of D and was accompanied by the formation of A and C. Saponification of some starting material eluted from the column gave A and benzoic acid. The I.R. spectrum (film) of the crude material showed absorption maxima at 1779 $cm.^{-1}$ and 1732 $cm.^{-1}$ consistent with aryl - O - alkyl and aryl - O - aryl esters respectively. Rapid column chromatography of the slightly contaminated D gave small amounts of TLC pure D. This proved to be identical in all

respects with dibenzoyl A. The benzoyalation of A had however to be carried out in two stages, first O - aryl benzoylation by Schotten-Baumann followed by benzoylation by the benzoyl chloride/pyridine method. The end product was a colourless viscous oil which slowly decomposed at room temperature. The mono benzoyl derivative of A (4-(p-benzoyloxyphenyl)-butan-2-ol) had an R_f similar to C and was the only compound in the A group series that crystallised. By careful TLC examination of alumina column chromatographic fractions, it was possible to crystallise compound C using seed crystals (from the synthetic product), and show its identity with the monobenzoyl A ,(4-(p-benzoyloxyphenyl)-butan-2-ol). Monobenzoyl A (by TLC) was also formed in the alumina column hydrolysis of the dibenzoyl A.

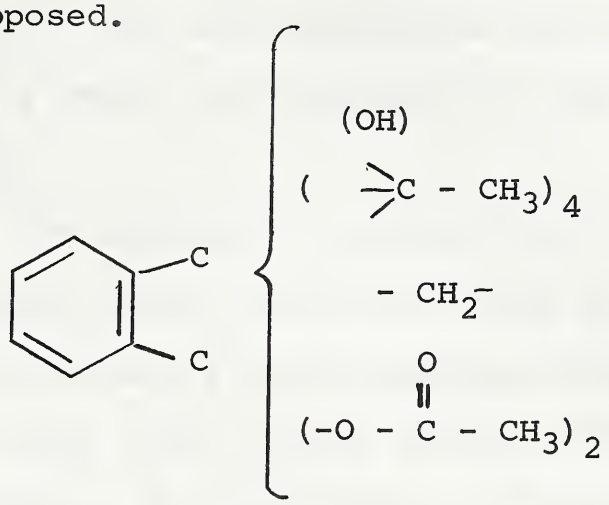
The natural occurrence and isolation of benzoate esters of relatively simple phenolic compounds is not common and has only previously been reported for Storax, Benzoin, Balsam of Peru and Balsam of Tolu. The latter are liquid or semisolid exudations and are composed chemically of benzoyl, toluyl and cinnamyl esters of phenol together with the free acids. 4-(p-hydroxyphenyl)-butan-2-ol has not previously been isolated in the free state although the 4-(p-hydroxyphenyl)-butan-2-O- glucoside has been reported present in Betula.alba (62) and Betula platyphylla (61).

Compound B was the last remaining material to be investigated since the yields of B were so small as to

preclude any systematic investigation. B was obtained in 30 mg. yield from 1.0 Kg of plant material; by the steam distillation method. It was also present in small amounts as a contaminant to A in alumina column chromatography of the E 3 series of extracts.

B was a colourless non-crystalline substance. I.R. spectrum (nujol mull) showed a bonded hydroxyl group 3340 $cm.^{-1}$ and a saturated ester carbonyl absorption band at 1730 $cm.^{-1}$ together with an absorption band at 710 $cm.^{-1}$ suggestive of an ortho disubstituted aromatic ring. The U.V. spectrum showed λ_{max} at 2033 Å, 2294 Å, 2739 Å and 2809 Å. There was no bathochromic shift in base, but the 2033 Å maximum shifted to 2132 Å. The lack of a bathochromic shift precludes free phenolic hydroxyl group. Permanganate oxidation of A with small amounts of B gave small amounts of phthalic acid. This established the presence of a 1:2 dicarbon substituted benzene ring or a polyaromatic system. The latter is considered unlikely due to the I.R. and U.V. spectral data. N.M.R. spectrum allowed assignment of the following groups. The three proton signals at 9.09 τ, 8.94 τ, 8.72 τ and 8.63 τ, are assigned to four methyl groups, all of them single peaks indicating that the methyl groups are attached to carbons carrying no hydrogen. A two proton signal at 8.23 τ (single peak) is assigned to a methylene group. Two, three proton signals at 7.96 τ and 7.92 τ are assigned to two acetyl groups. The four protons signal at 2.38 τ is assigned as four aromatic

hydrogens. Thus if one assumes a mono aromatic ring system is present, the following partial structure for B may be proposed.



Confirmation of this structure must however wait isolation and purification of further material followed by both chemical degradation and synthesis.

Compound F isolated from T. baccata, by the short path distillation of E7 and present as the major component was found to be identical to that of A from T. brevifolia and was therefore 4-(p-hydroxyphenyl) butan-2-ol. Compounds G and H were found to be the mono and dibenzoyl esters of F by TLC and hence identical to C and D from T. brevifolia. It is of interest to note that 4-(p-hydroxyphenyl)-butan-2-ol is present in very large quantities, which is greater than the alkaloidal complex "Taxine" fraction and it will always appear in the taxine fraction despite acid/base/neutral separation. It will therefore be a possible contaminant of 'taxine' using the isolation procedures of Graf (25-29) and also probably in that of Baxter

and coworkers (36, 37). The crude extract (E.7) containing F, G, H. from T. baccata would roughly correspond to the non-nitrogenous material millossan isolated by Amato and Capparelli in 1880 (11) from T. baccata.

The failure to isolate basic material from T. brevifolia under conditions which isolate an alkaloidal mixture from T. baccata refutes the recent claim of Tyler (50) to have isolated "Taxine" from T. brevifolia. Not even traces of basic materials showing the characteristic TLC patterns for "Taxine" could be detected. Furthermore it is extremely doubtful if 'taxine' could have survived the rigorous isolation and purification techniques of Tyler.

The present investigation does agree with Jones and Lynn (48) on the absence of alkaloids but volatile oil has been shown to be present. T. brevifolia extracts by paper chromatography do not show any of the sciadopitysin group of biflavanoidal pigments found in T. baccata. However preliminary examinations of the n-butanol extracts of the deep yellow aqeuous liquor left after extraction of the compounds described previously do show the presence of glucosides which have at least in part properties similar to the saponins.

CONCLUSION

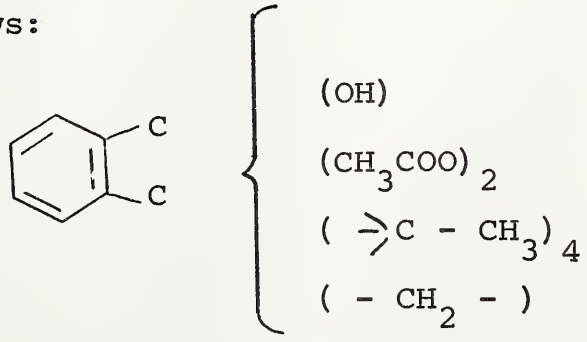
After a detailed investigation to establish the optimum conditions of extraction of the leaves and twigs of T. brevifolia, the soxhlet extraction using methanol was found to be the most efficient method. The most efficient preliminary separation of the components of the extract was effected using steam distillation. The final separation and purification was made possible by adsorption column chromatography and Thin Layer Chromatography. Through the application of these techniques three new compounds were isolated and their structures established by physical and chemical methods and finally confirmed by synthesis. These compounds were:

(1) 4-(p-hydroxyphenyl)-butan-2-ol

(2) 4-(p-benzoyloxyphenyl)-butan-2-ol

(3) 4-(p-benzoyloxyphenyl)-2-benzoyloxybutane

A fourth compound was also isolated in small amounts in a pure state. A partial structure for this compound based on both physical and chemical evidence is as follows:



Traces of a volatile oil(s) were also isolated, but not in sufficient quantities to permit a systematic chemical examination.

Alkaloids were not found in any specimen of *T. brevifolia*, although these were collected at different seasons and from different trees and localities. By contrast alkaloids were readily obtained from *T. baccata* using identical extraction procedures. In a preliminary study of *T. baccata* the non-alkaloidal constituents, 4-(*p*-hydroxyphenyl)-butan-2--ol and its mono and di-benzoyl esters were isolated for the first time.

The TLC pattern of the alkaloidal fraction ('Taxine') showed the presence of at least three major components.

PART III

EXPERIMENTAL

(i) GENERAL PROCEDURE

(a) Spectral data: The I.R. and U.V. spectra were recorded on Perkin Elmer D.B. 21 and Unicam S.P. 700 spectrophotometers respectively. The chloroform used for the spectral work was ethanol free, A.C.S. grade. Chloroform was chromatographed on silica gel column; the initial eluates were rejected and the following eluate used immediately (56). The ethanol used for the U.V. spectra was prepared as follows; I l. of 95% ethanol was refluxed with 1.0 g of sodium borohydride for two hours and then slowly distilled; the first 50 ml. of the distillate was discarded and the distillation continued until 700 ml had distilled. This fraction gave ethanol (95%), free from acetic acid and carbonyl impurities (56). N.M.R. spectra were obtained using Varian Associates A.60 and H.R. 100 spectrometers.

(b) Thin Layer Chromatography: Glass plates 20 cm x 20 cm and 10 cm x 8 cm were used. The plates were coated with the adsorbent by means of a Desaga SII adjustable applicator to give a uniform 0.25 mm layer. The adsorbents used were silica gel G and Kieselguhr G. supplied by Merck and company.

The majority of materials in this investigation were non-fluorescent on U.V. irradiation and the position of the separated components was shown by a 1% ceric sulphate in 10% sulphuric acid spray reagent (51). At room temperature

this spray reagent gave with some components, coloured spots. When the sprayed plate was heated to 100-110°C for three to five minutes all components gave deep brown to black spots on a white background. To locate the position of the separated materials on the thicker (1-2 mm) preparative plates a water spray was used (52) which gave white opaque bands on a translucent background. After marking their positions on the plate, the plate was dried and the bands were removed using a modified micro-vacuum cleaner technique (51, 53). Unless specifically stated the standard solvent system was n-hexane/chloroform/methanol (40:20:11). The reported R_f values are, for this solvent system with a solvent run of 8 to 9 cm, at 18-23°C, under the supersaturated chamber conditions (55).

(c) Adsorption chromatography:

Neutral alumina used, was supplied by M. Woelm Eschwege and was adjusted when necessary to a given Brockmann activity (54). The silica gel for adsorption chromatography was supplied by British Drug Houses, Ltd., Poole, England. The weight of the column material used was usually thirty-five times the weight of the sample to be chromatographed.

The solvent used in column chromatography were A.C.S. grade; the chloroform used was ethanol free and anhydrous. This was prepared as required: To A.C.S. grade chloroform was added A.R. fused calcium chloride. After

twenty-four hours the chloroform was filtered from the solid material and used as required.

(d) Paper chromatography:

The ascending paper chromatographic technique was used with Whatman No. 1 paper for chromatography.

(e) Plant material:

The leaves and small twigs of T. brevifolia Nutt, were collected in British Columbia, Canada, whilst T. baccata was obtained from Kashmir, India. The plant materials were obtained through the courtesy of Mr. Bev. Wendell, British Columbia and Mr. H. Handa of the Central Drug Research Institute, India.

(ii) EXTRACTION AND SEPARATION OF THE COMPONENTS OF T. brevifolia AND T. baccata.

(A) Extraction

Three general extraction procedures were used. They were:

(a) Cold percolation with 5% acetic acid in methanol.

(b) Cold percolation with 2% aqueous sulphuric acid.

(c) Soxhlet extraction using methanol.

Extraction of T. brevifolia by method (a):-

5.0 Kg of the powdered leaves and twigs were first percolated with n-heptane to exhaustion. The plant material was removed from the percolator and the excess heptane was allowed to evaporate. Percolation was then continued with 5% acetic acid in methanol until a portion of the percolate failed to give a residue on evaporation.

The heptane (15 l) and the methanol/acetic acid (20 l) percolates were separately evaporated "in vacuo" to a smaller volume (1 l). The deep green concentrated methanol/acetic acid extract was poured with stirring into 5 l of cold water. The initial turbid flocculent liquor so obtained separated into a green precipitate and a golden brown supernatant liquor, after standing at 0°C for twelve hours. The supernatant liquor was filtered through a Kieselguhr bed. The filtrate was basified with ammonia solution to pH 9-10. This solution was extracted with diethyl ether, until no further material was extracted with the solvent. The ether extract was washed with water and dried over anhydrous sodium

sulphate before evaporation under reduced pressure to a small volume (1 l). This was then extracted with cold, (4°C.), 1% sulphuric acid followed by 5% aqueous sulphuric acid. The acid extracts were made basic with ammonia to pH 9-10 and extracted with ether. The ether extracts were washed with water, dried and evaporated under reduced pressure to obtain 8.0 g of a yellow semisolid substance. This extract is numbered E.1.

Extraction of T. brevifolia by method (b):-

150 g of the finely powdered plant material were first percolated with n-heptane. After removing the heptane from the plant material, the percolation was continued with 2% aqueous sulphuric acid at 4°C. The percolate (800 ml) was basified with ammonia to pH 9-10 and exhaustively extracted with ether. The ether extracts were combined and washed with water. After drying, it was evaporated under reduced pressure to give 0.35 g of a yellow semisolid. Extract E.2.

Extraction of T. brevifolia by method (c):-

1.0 Kg of finely powdered plant material was first extracted with n-heptane in a soxhlet extraction apparatus, followed by methanol (A.C.S. grade). The heptane extract (12 l) and the methanol extract (12 l) were separately evaporated under reduced pressure to a smaller volume (1.25 l). The concentrated methanol extract was poured into five times its volume of cold 1% aqueous sulphuric acid and the resultant liquor was left overnight at 0°C. The dull green precipitate which separated was filtered off. The clear

filtrate was left for a further period of twelve hours at 0°C and again filtered free from a flocculent green precipitate. The clear filtrate was extracted with ether until a portion of the ether extract gave no residue on evaporation. The ether extract was washed with water and dried over anhydrous sodium sulphate. This gave, when evaporated under vacuum, at less than 30°C, a dark yellow viscous oil (18.0 g). This extract was extract E.3.

The aqueous liquor left after the ether extraction was made basic with ammonia (pH. 9-10) and further extracted with ether. This ether extract processed as above gave 0.55 g of a pale yellow semisolid. Extract E.4.

Small scale extractions (100. g) by method (c) of plant material collected in different localities at three different seasons, spring, summer, and fall, were made. These extracts are numbered E.3.a, E.4.a, E.3.b, E.4.b, E.3.c, and.E.4.c. No significant differences in these extracts could be detected.

Extraction of T. baccata by method (a):-

5.0 Kg of the powdered leaves and twigs were percolated with n-heptane until the percolate was colourless. The plant material was then freed from heptane and the percolation was continued with 5% acetic acid in methanol until a residue obtained from a sample of the percolate by evaporation gave a negative test with Mayer's reagent.

The heptane extract (15 l) and the methanol/acetic acid extract (20 l) were separately evaporated 'in vacuo ' to

a smaller volume (1.1 l). The concentrated methanol/acetic acid extract was poured into 5.0 l of cold water and left at 0°C overnight. It was then filtered free from the green precipitate using a bed of Kieselguhr. The clear yellow filtrate was made basic with ammonia to pH 9-10 and extracted with ether. The ether extract (10 l) after a water wash was evaporated to a smaller volume (1 l). This was extracted with 1% aqueous sulphuric acid until the extract gave a negative Mayer's test. The acid extracts were combined and ammonia added, to give a pH 9-10, and then extracted with ether. The ether extract after a water wash was dried over anhydrous sodium sulphate and evaporated under reduced pressure when 5.8 g of yellow semisolid material was obtained. This would correspond to the "taxine" alkaloidal complex. Extract E.5.

Extraction of T. baccata by method (b):-

500.g of the finely powdered leaves and twigs were percolated with 2% aqueous sulphuric acid until the percolate gave negative alkaloidal (Mayer's) test. The percolate (1.5 l) was brought to pH 9-10, with ammonia and was exhaustively extracted with ether. The combined ether extract was washed with water, dried over anhydrous sodium sulphate; then evaporated under reduced pressure to give 0.9 g of viscous yellow oil. Extract E.6.

Extraction of T. baccatta by method (c):-

1.1 Kg of the powdered leaves and twigs were extracted with n-heptane until the extract was colourless. The heptane extracted plant material was freed from heptane and the

extraction continued with methanol (A.C.S. grade) to exhaustion.

The heptane (12 l) and the methanol (12 l) extracts were separately concentrated under reduced pressure to a smaller volume (1 l). The deep green methanol extract (1 l) was poured into five times its volume of volume of cold 2% aqueous sulphuric acid and left overnight at 0°C. The deep green precipitate which collected at the bottom of the flask was filtered off and the golden yellow filtrate was extracted with ether to exhaustion. The ether extract was washed with water, dried over anhydrous sodium sulphate and evaporated under reduced pressure to give a thick viscous yellow liquid (15.0 g). Extract E.7.

The aqueous liquor remaining after the ether extraction was made basic with ammonia to pH 9-10, and again extracted with ether until a portion of the ether extract gave no residue on evaporation. The extract was then washed with water, dried over anhydrous sodium sulphate and evaporated under reduced pressure to give 5.4 g of a yellow semi-solid. Extract E.8. This extract gave positive Mayer's test and corresponds to "taxine".

(B) Evaluation of the extracts obtained from the various extraction procedures:-

At the start of the investigation the use of ascending paper chromatography using different solvent systems and spray reagents was attempted for the control and

qualitative identification of the individual components of the extract.

The following spray reagents were used with a test spot from the basic material (E.8) extracted from T. baccata.

Spray reagents	Colour reactions
1. 1% Aqueous potassium ferricyanide solution, the paper dried, then sprayed with 1%, aqueous ferric chloride.	Pale spot on a deep blue background.
2. 1% Aqueous phosphomolybdic acid solution.	No significant colour change
3. 1% Aqueous potassium permanganate solution.	Pale spot on a brown background after drying.
4. Lepage's reagent.	Canary coloured spot on pale yellow background.
5. Dragendorff's reagent (modified) (57).	Pale orange stain almost indistinguishable from the background.
6. 1% Aqueous solution of iodine.	Pale yellow spot on white background (after prolonged standing).

Reagents (1) and (3) were most successful and sensitive and were therefore used to evaluate the efficiency of the solvent mixtures in separating the test material.

Two general solvent systems were tried, after paper chromatography with simple solvent/water systems failed to give satisfactory separation.

1. Butanol/glacial acetic acid/water (4:1:5)
2. Butanol/formic acid/water (1:2:15)
3. Iso amyl alcohol/glacial acetic acid/n heptane/ water (3:3:1:2).

Whilst all the above solvent systems gave a reasonably compact spot R_f 0.8 - 0.85, no separation of the individual components was found. In spite of prolonged experimentation in varying both paper and solvent systems no separation of the individual components could be achieved. Identification of the components in the eluate fractions from the column chromatography of extract (E.3) had therefore to be accomplished by physical data. Only after it was found that this approach was less than ideal that a then little used technique, Thin Layer Chromatography was considered. Before this time little, if any information was readily available concerning its capabilities and of the experimental conditions required for its use.

Thin Layer Chromatography:-

Glass plates 10 cm x 8 cm coated with a 0.25 mm layer of silica gel G. were spotted with the extracts from T. brevifolia and T. baccata and also the eluates from the adsorption column chromatography of the extract E 3 (T. brevifolia). The prepared plates were developed under the supersaturated chamber technique of Stahl (55) with a solvent run of 8 - 9 cm. The plates were then removed from the chamber and allowed to dry at room temperature; fresh solvent was used for each run.

The following solvent combinations were tried.

1. Chloroform/n -hexane/diethylamine (10:20:3)
2. Chloroform/n -hexane/diethylamine (5:4:3)
3. Chloroform/n -hexane/methanol (5:10:3)
4. Chloroform/n -hexane/methanol (2:4:1)
5. Chloroform/n -hexane/methanol/diethylamine (10:20:3:2.3)
6. Chloroform/n -hexane/methanol (20:40:11)

Although solvent systems 1 - 4 gave a clear separation of some of the components of the total extract 5 and 6 proved to give a better overall separation. Solvent system 5 was initially used due to a somewhat sharper separation but was found later, to have disadvantages for use in preparative TLC (due to the diethylamine). No. 6 was thereafter used as the standard solvent and all the TLC results are reported, unless otherwise stated for this solvent. The R_f values of a given component in 5 and 6 did not show any significant differences.

Extracts obtained from T. brevifolia E.1, E.2, and E.3 gave identical chromatograms; whereas E_4 showed the absence of at least one component. The major component appeared as a violet spot on the chromatograms, when sprayed with the ceric sulphate/sulphuric acid reagent, at room temperature, whereas the other components appeared as dark spots when the chromatographic plate was heated to 100-110°C for five minutes. The different extracts, the components present in them and their R_f values are listed below. For the convenience of discussion the components are for the present referred to as compound A, B, C, D, E etc. Components with

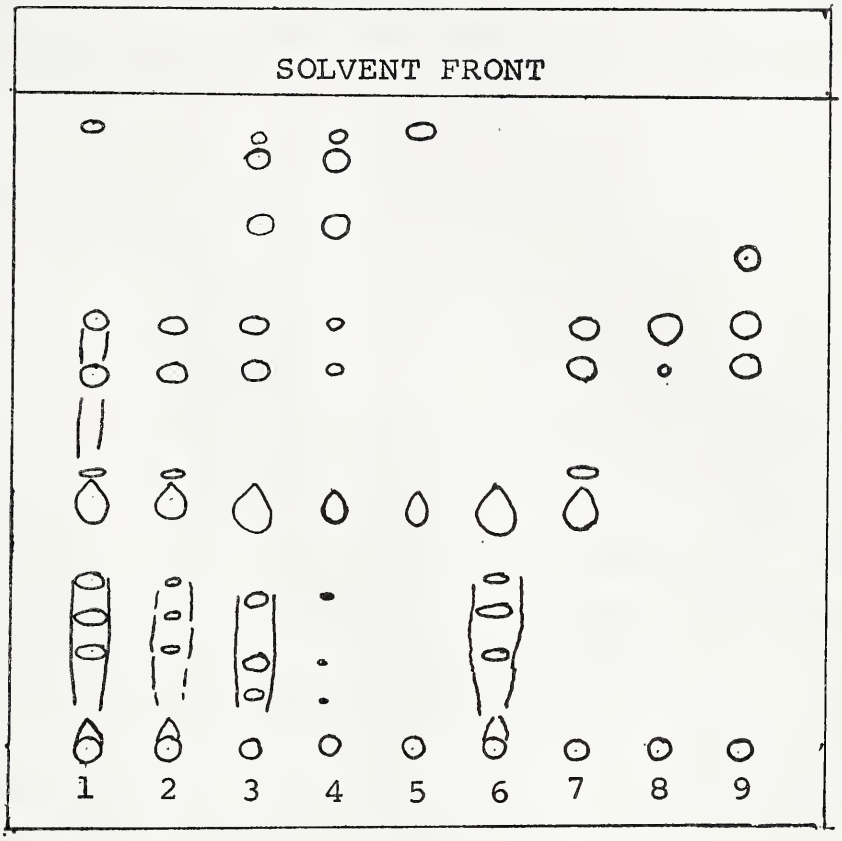
R_f values less than 0.38 did not give satisfactory separation except at low concentrations. These constituents were not further investigated.

The various extracts from T. brevifolia, the components and their R_f values.

Extract	Components	R_f	Comments
E.1	A	0.38	Major component
	B	0.41	Minor component
	C	0.58	Minor component
	D	0.66	Minor component
	E	0.96	Only in very small amounts.
E.2	A,B,C,D,E.	same as given above for E.1.	A is the major component. The other components are present in the same extent as E.1.
E.3	A,B,C,D,E.	Same as given for E.1	A is the major component. The other components are present in the same extent in E.1 and E.2.
E.4	A,B,C,D,	R_f values are same as for E.1	A is the major component. All others are present in small amounts.

E.1, E.2 and E.3 contained three components with R_f values less than 0.38. Those components had R_f values 0.25, 0.2 and 0.15. All the E.3 series had similar if not identical chromatograms as had all the E.4 series. The components with R_f less than 0.38 were present only in very small amounts in the E.4 series.

TLC of the Extracts E3, E4, E7, E8, the different fractions of the steam distillation products of E3 and the synthetic derivatives of Compound A.



1. Extract E3
2. Extract E4
3. Extract E7
4. Extract E8
5. Steam Distillate extracted with ether
6. Supernatant liquor left in the steam distillation flask
7. Cold benzene extract of the resinous residue left in the steam distillation flask
8. Hot benzene extract of the resinous residue left in the steam distillation flask
9. Monobenzoyl derivative of A
   Dibenzoyl derivative of A
   Diacetyl derivative of A

TLC examination of the extracts from T. baccata

Extracts E.5, E.6, E.7, and E.8 were examined by TLC in the same manner as above. The results of the chromatogram are summarised in the following table.

Extract	Components	R_f	Comments
E.5	F	0.38	Major component
	G	0.58	Minor component
	H	0.66	Minor component
	I	0.81	Pink-when sprayed with the spray reagent at room temp.
	J	0.88	Yellow-when sprayed with the spray reagent at room temp.
	K	0.92	Orange-red -when sprayed with the spray reagent at room temp.
E.6	F,G,H,I,J, K.	same as in E.5	Same as in E.5.
E.7	F,G,H,I,J, K.	Same as in E.5	Same as in E.5 I,J, and K present in traces.
E.8	F,G,H,I,J, K.	same as in E.5	The relative concentration of I,J,K, is much higher than in E.7.

Thus all the extracts from T. baccata had components F, G, H whose R_f corresponded to the three components A, C, and D, found in T. brevifolia. The three components I, J, and K which were present only in trace amounts in extract E.7, were however, present in relatively larger quantities in E.8; these may represent at least in part the individual components of the "Taxine" alkaloidal complex. In addition there was a complicated series of spots, whose R_f were below

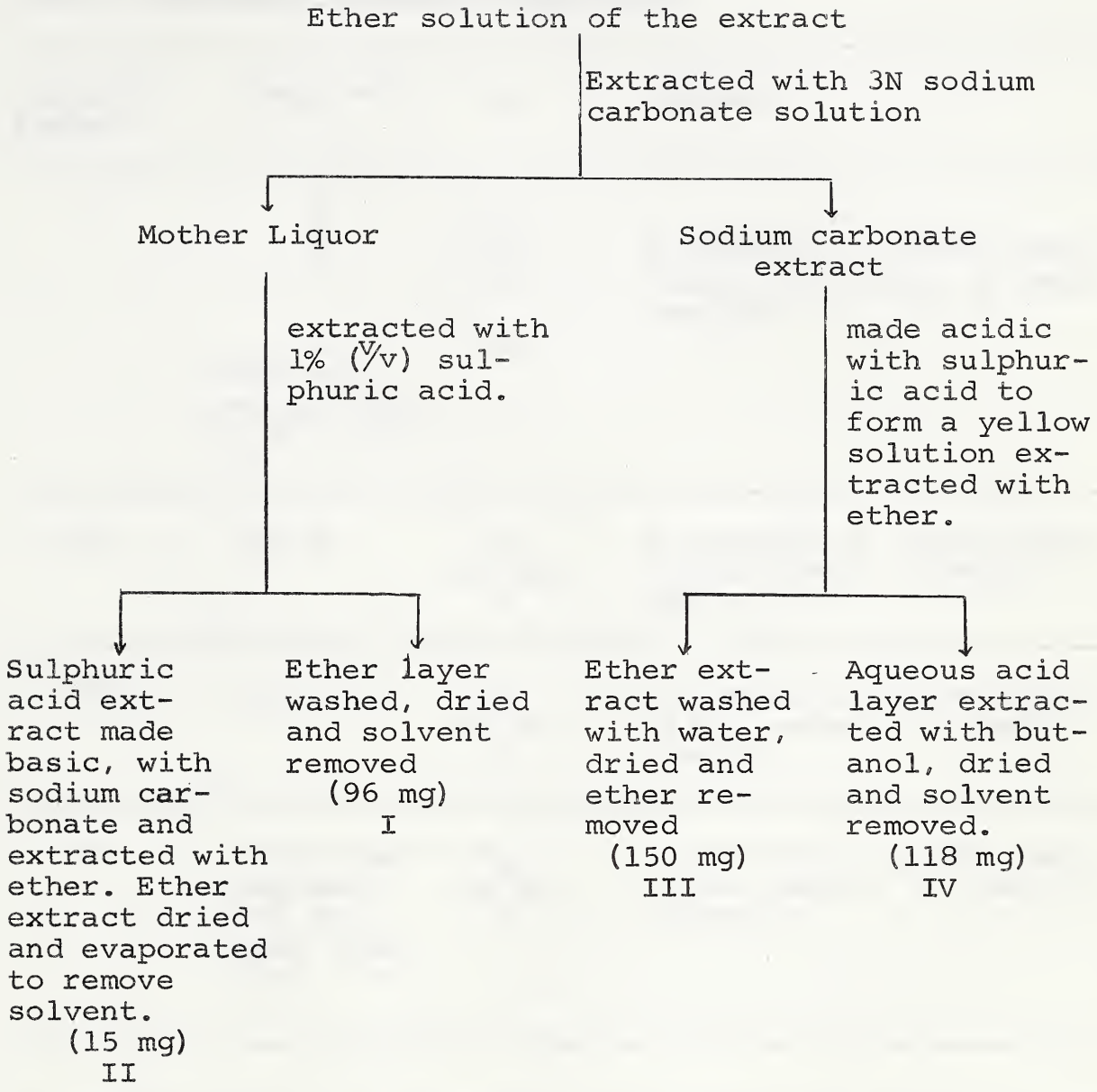
0.38, which could only be resolved into three components when in low concentration. Experimentally one would expect polyhydroxy compounds, under the conditions used, to have low R_f values. These components may then represent the deaminated and deacetylated Taxus alkaloids.

(C) Fractionation of extract E.3 of T. brevifolia:-

(a) Acid base, neutral separation:-

The extract E.3 gave positive Mayer's test. Since it was possible that very weak bases might have been extracted with the acidic and neutral fractions an acid/base/neutral separation was carried out.

1.0 g of extract E.3 was dissolved in the minimum volume of ether and the separation was carried out according to the following scheme. The separated fractions were then examined by TLC.


Ether solution of the extract
Extracted with 3N sodium carbonate solution
Mother Liquor
Sodium carbonate extract
extracted with 1% (v/v) sulphuric acid.
made acidic with sulphuric acid to form a yellow solution extracted with ether.
Sulphuric acid extract made basic, with sodium carbonate and extracted with ether. Ether extract dried and evaporated to remove solvent.
(15 mg)
II
Ether layer washed, dried and solvent removed
(96 mg)
I
Ether extract washed with water, dried and ether removed
(150 mg)
III
Aqueous acid layer extracted with butanol, dried and solvent removed.
(118 mg)
IV

TLC of different isolated fractions:-

Fraction Number	Components	R_f	Comments
I	A	0.38	
	B	0.41	A present in large quantities and others in small amounts
	C	0.58	
	D	0.66	
	Some compounds with $R_f < 0.38$		
II	A,C,D.	R_f as in I	A present in large quantities, others in small amounts
III	A,B,C.	R_f as in I	A present in large quantities, others in small amounts.
IV	A,C,D and compounds with Rf lower than 0.38	R_f as in I	A present in large quantities, others in smaller amounts.

In addition the I.R. spectra of the different fractions did not show any significant differences.

(b) Short path distillation:-

1.8 g of the extract E 3 was subjected to a short path vacuum distillation/sublimation. After heating at 40°C for two hours two distinct zones were present on the cooled portions of the tube used for the distillation. The zone, further away from the heating block consisted of a white

crystalline compound and the other zone was present as a colourless viscous oil. Both zones were separated by cutting the tube. The residual non-distilled material was also recovered.

The white crystalline substance (25 mg) had a m.p. 120.5-121°C and gave a positive acid test with saturated sodium bicarbonate solution. The U.V. and I.R. spectra were identical in all respects with that of benzoic acid. A mixed melting point with benzoic acid showed no depression.

Analysis; Found C = 68.9 %, H = 4.88 %; calculated for $C_7H_6O_2$; C = 68.84 %, H = 4.95 %.

The viscous liquid was examined by TLC and found to be identical with the major component A, (R_f 0.38) with very small traces of compound D. Attempts to crystallise the oil from organic solvents failed. This oil on standing at 0°C for several weeks or when cooled to - 70°, in a dry ice/acetone bath, solidified as a crystalline mass m.p. 65° - 68°C.

The residue from the distillation was recovered and TLC indicated that it contained further amounts of compound A, (R_f 0.38) and components not seen in the sample before distillation.

(c) Separation by adsorption column chromatography:-

Column I:- 2.0 g of the extract E 3 from T. brevifolia was chromatographed on a 70 g, alumina, activity V column. Since E 3 was not completely soluble in benzene or heptane, the extract was dissolved in chloroform/benzene (1:4) and was then placed on the column. The column eluates were collected in 15 ml fractions. The elution pattern was followed by the weight/fraction and TLC techniques.

Column I.

Solvent	Fraction Numbers	Weight of fractions (mg)	TLC
Chloroform/benzene (1:4)	(1-18)	63	E,D,C.
Chloroform/benzene (1:1)	(19-70)	98.9	A,B.
Chloroform/benzene (3:1)	(71-83)	53.6	A,B, Compounds of lower R_f.
Chloroform	(84-97)	79.5	A,B, Compounds of lower R_f.
Methanol/chloroform (0.5:99.5)	(98-109)	56.6	A,B, Compounds of lower R_f.
Methanol/chloroform (2:98)	(110-135)	258.8	A,B, Compounds of lower R_f.
Methanol/chloroform (5:95)	(136-145)	23	A, Compounds of lower R_f
Methanol/chloroform (10:90)	(146-155)	15.4	A, Compounds of lower R_f.
Methanol	(156-160)	10	A, Compounds of lower R_f.

Fractions (19-70) after removing the solvent gave a colourless viscous liquid. This viscous liquid after standing at $0^{o}C$. for several weeks or when cooled in a dry ice/acetone bath solidified to a crystalline mass. But attempts to crystallise it from solvents failed. TLC showed it to be compound A with traces of compound B.

Column II

7.0 g of the extract E 3 from T. brevifolia were chromatographed on 245 g of a column made from alumina (activity V). The eluate was collected in 15 ml fractions. In this case E 3 was poured on to the top of the column and not in solution of any solvent. The column was eluted with solvents of increasing polarity as before. The elution pattern was followed as for column I, but only alternate fractions were examined by TLC. Fractions were combined on the basis of TLC patterns.

Solvent	Fraction Numbers	Weight of fractions (mg)	TLC
Benzene	(1-42)	27.8	E,D.
Chloroform/benzene (1:1)	(43-58)	30.4	E, with more D.
Chloroform/benzene (1:1)	(59-89)	222	C,D,E.
Chloroform/benzene (1:1)	(90-99)	31	A,B,C,D, A in small amount
Chloroform/benzene (4:1)	(100-120)	606.6	A,B,C,D. A predominating
Chloroform/benzene (4:1)	(121-140)	150.1	A,B. B in traces.
Chloroform	(141-152)	1275.6	A,B, B in traces.
Stripped the column with chloroform/ methanol (98.5:1.5)	(153)	1287.0	A,B. B in traces.
Stripped the column with methanol	(154)	560	A,B compounds with R_f lower than 0.38.

Just as in column I fractions (131-153) were combined and when evaporated gave a pale coloured viscous liquid. This corresponds to fractions (19-70) of Column I. It did not crystallise from solvents, but on standing at $0^{\circ}C$. for several weeks solidified.

(d) Steam distillation of E.3:-

7.5 g of E.3 from T. brevifolia were steam distilled. The distillate and the residue left in the flask were further processed as follows.

Distillate:

The steam distillate (1.0 l) was extracted exhaustively with ether. The ether extract was washed with water and then dried over anhydrous sodium sulphate. The dried ether extract (400 ml) was distilled in batches of 50 ml through a Fenske ring fractionating column. The material distilled at 35°C was found to be completely diethyl ether. When all the ether was distilled the distillation was stopped. The residue left in the distilling flask (10 mg) was a yellow volatile liquid having a peculiar odour. TLC showed this to be compound E with traces of A. Compound E was previously shown by TLC, to be present in trace amounts, in the extract E.3.

Residue:

The residue after steam distillation was composed of a yellow supernatent liquor over a brown resinous mass. The yellow liquor was decanted and extracted with ether until the ether extract was colourless. The ether extract was washed with water, dried and the ether removed under reduced pressure to give 3.5 g of a viscous oil. TLC of this oil showed that it was mostly of compound A along with compounds of lower R_f 0.25, 0.2 and 0.15.

The brown resinous mass was dissolved in chloroform. This solution was dried and the chloroform removed under reduced pressure. The resultant viscous mass was first extracted with cold and then with hot benzene. Removal of the benzene under reduced pressure gave 1.3 and 0.20 g of residue respectively. These fractions are called benzene extract (1) and (2) respectively and neither benzene extract (1) nor (2) crystallised.

TLC of the different fractions obtained by steam distillation procedure

Fractions	Components	R_f	Comments
Fraction isolated from the distillate.	E	0.96	
	A	0.38	
Fractions recovered from the supernatent liquor.	A	0.3	Primarily of A
	Compound of lower R_f		
Benzene extract I.	A	0.38	
	B	0.41	D in trace amounts
	C	0.58	B and C enriched than
	D	0.66	in Extract E.3.
Benzene extract 2.	C	0.58	C present only in very
	D	0.66	small traces

Column chromatographic separation of the components in benzene extract I.

Column III.

Benzene extract I. was chromatographed on a column composed of alumina, (45.5 g), activity $\overline{V}$. 10 ml. eluate fractions were collected and the progress of separation followed as in the previous columns.

Column $\overline{III}$

Solvent	Fraction Number	Weight in mg.	TLC
Benzene	(1-12)	211.5	B,C, and A in traces
Benzene	(13-26)	180.9	A,B, and B in traces
Benzene/chloroform (4:1)	(27-32)	32.8	A,B, B in traces.
Chloroform (ACS)	(33-34)	420	A, B in traces.

Fractions (1-12) were combined and were then rechromatographed on a 7.4 g alumina, activity $\overline{V}$, column. 5 ml. eluate fractions were collected.

Column IV

Solvent	Fraction Number	Weight in mg	TLC
Benzene	(1-3)	3.3	C, B, B in traces
Benzene	(4)	66.8	B,C, C in traces
Benzene	(5-8)	25.6	A,B,C.
Chloroform	(9-10)	21.3	A,B,C.

Fractions (1-3) of column IV consisted mostly of compound C with traces of compound B. Eluate fraction 4 consisted mainly of B with some C. The other fractions were composed of A, B, and C.

Eluate fraction 4 of Column IV was rechromatographed on a silica gel, (2.4 g), column. 5 ml eluate fractions were collected. The progress of separation on the column was followed as before.

Column $\overline{V}$

Solvent	Fraction Number	Weight in mg	TLC
Benzene	I	1.5	B
Benzene	2	10.5	B
Benzene	3	3	B
Chloroform/ Benzene (1:1)	4	30	B,C.
Chlorform	5	10	B,C.

Fractions (1-3) of column $\overline{V}$ were TLC pure compound B.

Purification of benzene extract 2.

0.15 g of benzene extract 2 was chromatographed on a column composed of 0.515 g of alumina (activity $\overline{V}$). 5 ml eluate fractions were collected.

Column VI

Solvent	Fraction Number	Weight in mg.	TLC
Benzene	1	3	D
Benzene	2	4	D,C,A.
Benzene	3	10	D,C,A.
Benzene/ Chloroform (4:1)	4	17.8	D,C,A.
Stripped the column with chloroform (ACS grade)	5	120	D,C,A.

As the progress of separation on Column VI was followed by TLC it became apparent that compound D was undergoing chemical change. Further chemical work on D is reported in part III , Section (iii).

(e) Short path distillation of extract E.7 (T. baccata)

1.0 g of the extract was distilled in the same manner as with extract E.3. This also resulted in two zones, the components of which were benzoic acid (10 mg) and a colourless viscous oil (40 mg).

TLC of the viscous oil showed that it contains mainly compound F. whose R_f (0.38) was identical to that of A from T. brevifolia, and Compound H, whose R_f (0.66)

corresponding to compound D from T. brevifolia. The oil was therefore chromatographed on an alumina column, (1.2 g).

Column VII

Solvent	Fraction Number	Weight in mg.	TLC
Benzene	1	2	H
Benzene	2	3.5	F and H in traces
Benzene	3	4	F
Benzene/chloroform (1:1)	5	10	F
Column flushed with chloroform (A.C.S. grade)	6	10	F

Fractions (3-6) were combined and the solvent removed under reduced pressure to obtain a colourless viscous liquid. The I.R. spectrum, U.V. spectrum and N.M.R. spectrum of this substance was identical to that of compound A obtained from T. brevifolia.

(iii) CHEMICAL EXAMINATION OF THE ISOLATED COMPOUNDS

Compound A:-

Preparation of an analytical sample:-

Pure A was obtained by preparative TLC from elute fractions (19-70) of Column I. These combined fractions contained A as the major component, but had traces of B present. The preparative plates 20 cm x 20 cm were coated with 1 mm. layer of silica gel G. A methanol solution of eluate fractions (60 mg) was applied as a straight band across the plate 1 cm from the bottom of the plate. The plates were developed under the supersaturated chamber condition, using the solvent system n-hexane/chloroform/methanol/diethylamine (20:10:3:2.3); for a solvent run of 16 cm. After development, which took approximately 100 minutes, the plates were removed and the excess solvent on them allowed to evaporate at room temperature. The plates were then sprayed with distilled water; irradiated with U.V. light, when two bright violet white zones were visible on a pearly translucent background. The zones were marked and the plates allowed to dry. When dry, the marked zones were removed by the "micro-vacuum cleaner" technique. Elution of the so recovered silica gel with methanol followed by evaporation under reduced pressure gave from the broad band 55 mg. of TLC pure A. The other minor band was not eluted at this time. The A so obtained did not crystallise from organic solvents, but remained as a viscous liquid. The viscous liquid was dried

'in vacuo' for twenty-four hours. This then gave the following analytical values.

Found: C = 66.3%, H = 7.67%, N = 2.09%, calculated for $C_{37}H_{51}NO_{10}$; C = 66.37%, H = 7.67% and N = 2.09%.

25 mg. of compound A with traces of B obtained from eluate fractions (19-70) from the alumina column I was processed by the preparation TLC method as above but the development of the plate was carried out in n-hexane/chloroform/methanol (40:20:11). The detection of the bands and the isolation of A as described previously resulted in 20 mg. of TLC pure A. The physical characteristics were as before. It gave the following elemental analysis. Found C = 70.33%, H = 8.17%, N = 0.00. Molecular weight 170, as determined by vapour pressure method using Mechrolab, Model 301A osmometer, and 140 by Rast method, calculated for $C_{10}H_{14}O_2$; C = 72.3%, H = 8.43%.

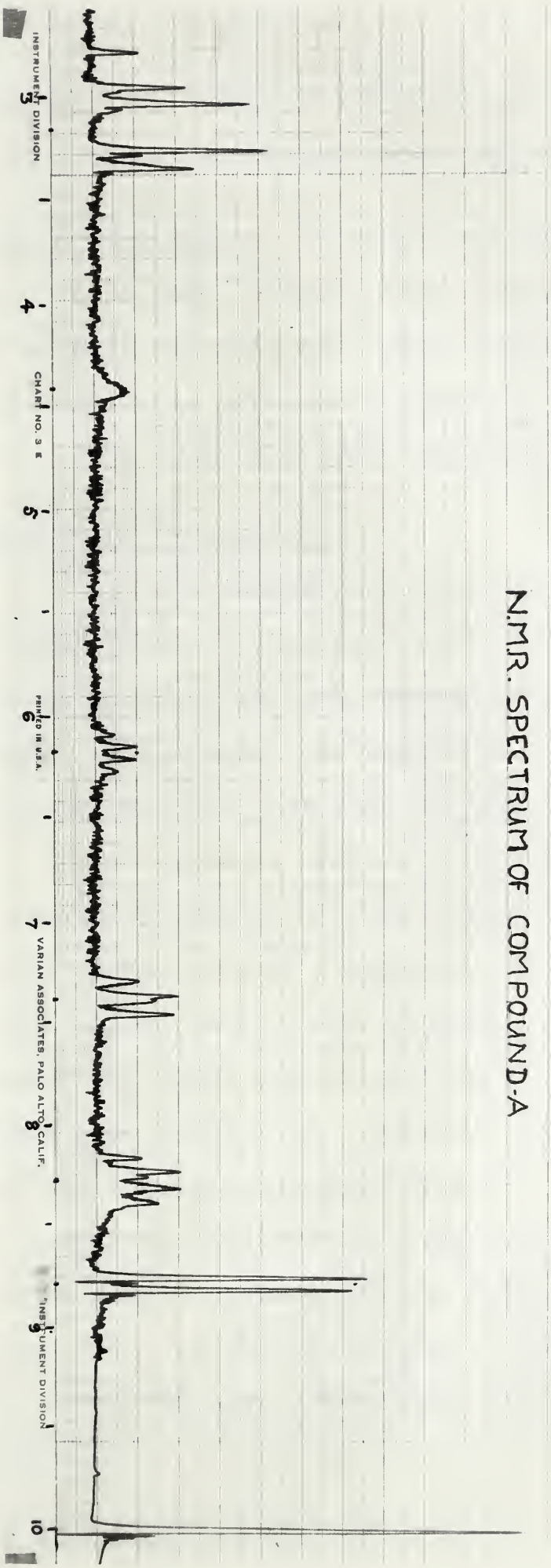
ν_{max} (film) 3275 $cm.^{-1}$ (OH) 1610 $cm.^{-1}$, 1590 $cm.^{-1}$ 1510 $cm.^{-1}$ (C = C aromatic), 824 $cm.^{-1}$ (p-disubstitution)

ν_{max} (KBr). 3350 $cm.^{-1}$, (OH) 1610 $cm.^{-1}$, 1590 $cm.^{-1}$, 1510 $cm.^{-1}$, 850 $cm.^{-1}$, 835 $cm.^{-1}$, 820 $cm.^{-1}$, 800 $cm.^{-1}$.

λ_{max} and λ_{max} (acid): 2033 Å ($\log\epsilon$ = 3.74), 2247 Å ($\log\epsilon$ = 3.87), 2800 Å ($\log\epsilon$ = 3.77)

λ_{max} (base): 2118 Å ($\log\epsilon$ = 3.82), 2427 Å ($\log\epsilon$ = 3.80) 2966 Å ($\log\epsilon$ = 3.79)

N.M.R. spectrum - 3.15 τ (4 aromatic protons) 4.4 τ (1 proton - CH (O<u>H</u>)), 6.19 τ (1 proton - CH_3.C<u>H</u>-)



N.M.R. SPECTRUM OF COMPOUND-A

7.37 τ (2 protons - $C\underline{H}_2$-) 8.27 τ (2 protons - $C\underline{H}_2$-)
8.78 τ (3 protons, twin peak -CH - $C\underline{H}_3$).

Physical properties:- Soluble in methanol, ethanol, acetone, chloroform, diethyl ether, benzene, and partially soluble in water and dilute mineral acids, completely soluble in aqueous alkaline solution (pH > 8) m.p. 64 - 68°C after previous cooling to -70°C.

Acetylation of Compound A:-

0.13 g of compound A with traces of B obtained from column II, eluate fractions (131 - 140), was dissolved in 5 ml of pyridine and was treated with 10 ml of acetic anhydride. The mixture was heated on a water bath for twelve hours and was then left at room temperature overnight. Excess pyridine and acetic anhydride were removed under reduced pressure. To the syrupy residue water was added and extracted with chloroform. The chloroform extract was washed with dilute sulphuric acid, then water, and evaporated under reduced pressure to give 0.14 g of a viscous pale yellow oil. This was dissolved in methanol (10 ml) and the solution decolourised with charcoal. After filtration, followed by evaporation of the filtrate 0.118 g of a pale viscous oil was left. This did not crystallise. TLC of the oil showed it had three components, R_f 0.6, 0.75, and 0.9. The compound R_f 0.75 gave the major spot.

Purification of the acetylated compound:-

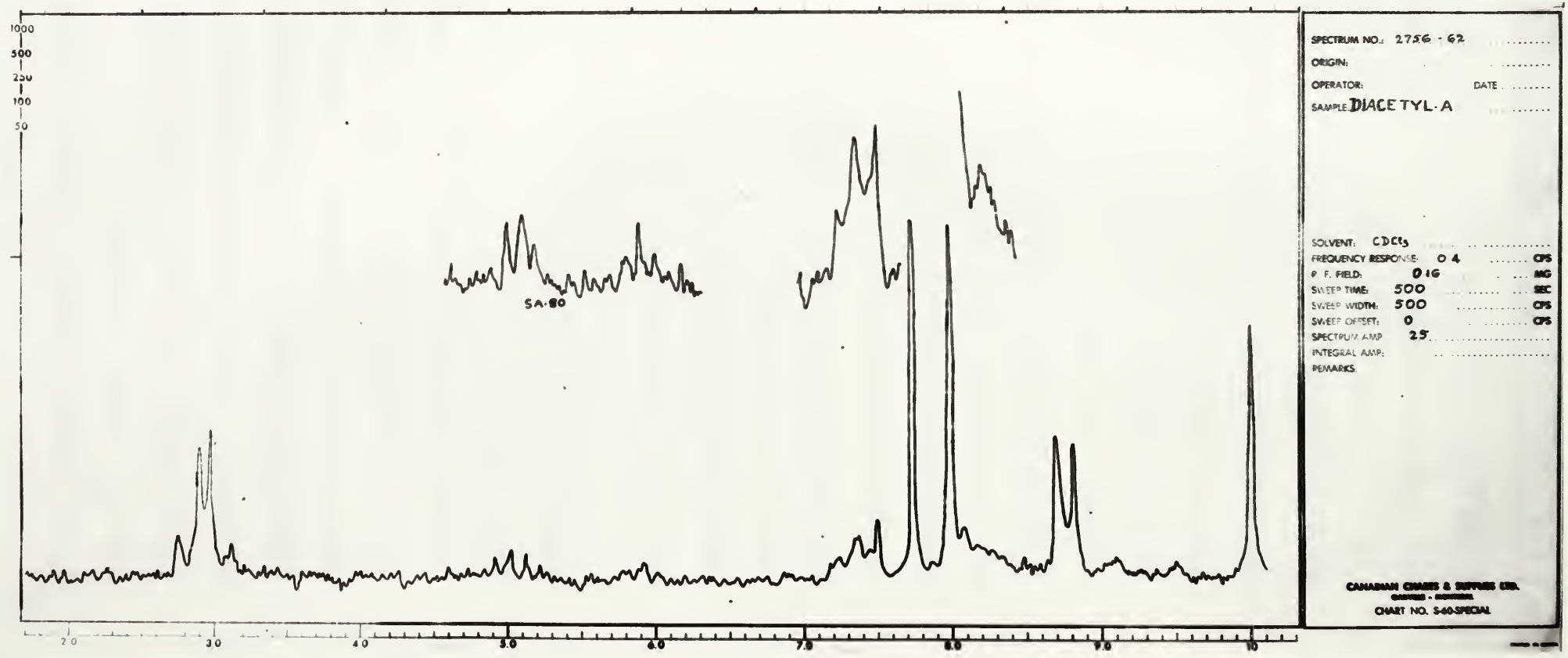
The acetylated product 0.118 g was chromatographed on 4.1 g of alumina (activity V) column. The eluate fraction volume was 10 ml.

Column VIII

Solvent	Fraction Number	Weight in mg.	TLC (R_f)
Benzene	1	1.9	0.9; 0.75
Benzene	2	69.4	0.75
Benzene	3	10	0.75, 0.6
Benzene	4	0.9	0.38, 0.4

Fraction 2, was TLC pure acetylation product of A. It was also a thick noncrystallisable viscous liquid. The R_f value was 0.75 in contrast to A, whose R_f = 0.38. The acetylated product gave the following analysis. Found C 66.91%; H 7.01%; Molecular weight 258; $-OCCH_3$, 37.46%; calculated for $C_{14}H_{18}O_4$ C, 67.26%; H, 7.26% $-OCCH_3$ (2), 34.4%.

λmax and λmax (acid): 2101 Å ($\log \epsilon$ = 3.62), 2162 Å ($\log \epsilon$ = 3.69), 2645 Å ($\log \epsilon$ = 2.45) 2703 Å ($\log \epsilon$ = 2.36) On a rapid scan, when base was added to solution of A, there was no shift in λmax, but a rapid change took place giving the following.


1000
500
250
100
50
SA-80
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
SPECTRUM NO.: 2756 - 62
ORIGIN:
OPERATOR:
DATE
SAMPLE: DIACETYL-A
SOLVENT: CDCl3
FREQUENCY RESPONSE: 0.4 CPS
R. F. FIELD: 0.16 MG
SWEEP TIME: 500 SEC
SWEEP WIDTH: 500 CPS
SWEEP OFFSET: 0 CPS
SPECTRUM AMP: 2.5
INTEGRAL AMP:
REMARKS:
CANADIAN CHARTS & SUPPLIES LTD.
CHART NO. S-60-SPECIAL

λ_{max} (base): 2100 Å ($\log\epsilon$ = 3.56), 2430 Å ($\log\epsilon$ = 3.72) 2955 Å ($\log\epsilon$ = 3.97).

ν_{max} (film) 1765 $cm.^{-1}$ (aryl ester) 1735 $cm.^{-1}$ (ester) 1510 $cm.^{-1}$ (C = C aromatic skeletal in plane vibration).

N.M.R. spectrum: 2.9 τ (4 aromatic protons) 5.4 τ (1 proton = C<u>H</u>·(OAc), 7.3 τ (2 protons - $C\underline{H}_2$ -), 8.2 τ (2 protons - $C\underline{H}_2$ -) 7.75 (3 protons of -O.CO $C\underline{H}_3$) 7.95 τ (3 protons of -OCO $C\underline{H}_3$) 8.77 τ (twin peak, 3 protons -CH - $C\underline{H}_3$)

Hydrolysis of diacetyl A.

15 mg of diacetyl A (TLC pure) was dissolved in 3 ml of methanol and was treated with 1.5 ml of sodium methoxide solution. The mixture was allowed to stand at room temperature for two hours. Then it was neutralised with dilute sulphuric acid; the reaction mixture was evaporated under reduced pressure at room temperature, the residue was extracted with chloroform, the chloroform extract was washed with water, dried over anhydrous sodium sulphate and evaporated to a small volume. TLC showed only A, R_f 0.38. The oil obtained by the complete evaporation of the chloroform had the physical characteristics of A. This confirmed that A did not undergo any rearrangement on acetylation.

Lead tetraacetate oxidation of A and diacetyl A:-

The sample used for this oxidation was one of the fractions isolated from the alumina column, which contained only very small traces of B. For this reaction the following solutions were made.

1. A saturated solution of lead tetraacetate in glacial acetic acid at room temperature (20°C) diluted five times with glacial acetic acid. 2. 3% potassium iodide solution in a saturated solution of sodium acetate in water, N/100 sodium thiosulphate and N/100 iodine solution.

31.6 mg of the sample were dissolved in the minimum quantity of glacial acetic acid, was treated with 10 ml of the above lead tetraacetate solution, and the volume made up with glacial acetic acid to 25 ml in a volumetric flask. A blank consisting of 10 ml of the lead tetraacetate solution made up to 25 ml with glacial acetic acid in a volumetric flask. At regular intervals 1 ml of the blank and 1 ml of the reaction mixture were added separately to 10 ml of the potassium iodide in sodium acetate solution. After five minutes 10 ml. of N/100 sodium thiosulphate were added to each of them. The excess sodium thiosulphate was then titrated with N/100 iodine using starch solution as indicator. The results of the experiment are given below.

Time in Hours	Volume of N/100 iodine consumed by blank (ml)	Volume of N/100 iodine consumed by sample (ml)	Difference
1	7.4	9.3	1.9
2	7.4	9.3	1.9
24	7.4	9.3	1.9

Amount of lead tetraacetate consumed by one mole of compound was 5 moles for $C_{37}H_{51}NO_{10}$. Subsequent work showed that the correct formula was $C_{10}H_{14}O_2$, consumption was then 1.25. When diacetyl A was subjected to oxidation under the above conditions for A, after 72 hours there was only 0.01 molar consumption of lead tetraacetate.

Hydrogenation of compound A:-

9.5 mg of TLC pure A were quantitatively hydrogenated at atmospheric pressure (697.5 mm/Hg) and at room temperature 20°C., using Adam's catalyst (10 mg) as catalyst. The hydrogenation was carried out in a 5% solution of sulphuric acid in 95% ethanol. 3.2 moles of hydrogen were consumed during a period of three hours. Even after twenty-four hours there was no further consumption of hydrogen. When there was no more further consumption of hydrogen the reaction mixture was treated with barium carbonate to remove the sulphuric acid. The precipitated barium sulphate and excess barium carbonate were filtered off and the filtrate evaporated. The residue after evaporation was subjected to TLC when no component of R_f 0.38 (ie A) could be detected. A broad oval streak $R_f > 0.4$ was produced.

Qualitative chemical tests on A:-

Formation of azo dye:-

To a solution of 20-30 mg of aniline in dilute hydrochloric acid at -10°C was added 2 ml of 10% aqueous sodium nitrite solution also at -10°C.

This mixture was added to a cooled solution of compound A (10 mg) in dilute sodium hydroxide. An orange red precipitate was formed immediately, indicating the presence of a phenolic hydroxyl group in A.

Iodoform test:-

50 mg of A were dissolved in 2 ml of a 12% aqueous solution of sodium hydroxide. To this solution was added dropwise a potassium iodide/iodine solution; until there was slight excess of iodine (pale brown colour). The mixture was then heated on a hot water bath. As the colour of iodine disappeared more of iodine solution was added until the colour of iodine persisted for a few minutes. A few more drops of 12% sodium hydroxide solution were added until the brown colour just disappeared. Then to the hot solution was added about 10 ml of water. A yellow precipitate was formed. This had the odour of iodoform and melting point and mixed melting point with iodoform 119.5-120°C.

Synthesis of A

(a) Synthesis of 4-(p-hydroxyphenyl)-3-buten-2-one (I)

This compound was synthesised according to Zemplén (59). 4.8 g of p-hydroxybenzaldehyde, 18 ml of acetone and 14 ml of a 12% aqueous solution of sodium hydroxide were mixed and shaken for twenty-four hours. The orange-red crystals deposited were collected, dissolved in water and acidified with 10% hydrochloric acid. On acidification an oily liquid separated, which solidified after keeping at 0°C. for three hours. The solid (3.5 g) after crystallisation and recrystallisation

from hot water, gave 2.5 g of pale yellow crystals m.p. 110.5°C. - 111°C. Reported 112 - 113°C. (59) and 112°C. (60). TLC. R_f 0.60.

ν_{max} 3125 $cm.^{-1}$ (-OH intermolecularly bonded), 1670 $cm.^{-1}$ (-CH=CH-C(=O)-), 1630 $cm.^{-1}$ (-C=C-phenyl), 1600 $cm.^{-1}$ and 1515 $cm.^{-1}$ (-C=C-aromatic), 815 $cm.^{-1}$, 835 $cm.^{-1}$ (p-substituted).

λ_{max} and λ_{max} (acid), 2340 Å ($\log \epsilon$ = 3.84), 3270 Å ($\log \epsilon$ = 4.26).

λ_{max} (base), 2520 Å ($\log \epsilon$ = 3.78), 3250 Å ($\log \epsilon$ = 3.4), 3900 Å ($\log \epsilon$ = 4.39).

(b) Synthesis of 4-(p-hydroxyphenyl)-butan-2-one (II)

1.0 g of (I) was hydrogenated in 95% ethanol, using palladium/charcoal (150 mg) as catalyst. When hydrogen uptake ceased the hydrogenation was stopped and the catalyst was filtered off from the reaction mixture and the filtrate was evaporated to dryness under vacuum to give 1.0 g of the hydrogenated product. This was not purified and was used directly in the next step of the reaction. TLC revealed two spots R_f 0.38 and 0.43. The hydrogenation was repeated using 0.50 g. This resulted in a reaction product which contained two components, R_f 0.38 and 0.43.

Zemplén (59) reported the reaction product as a crystalline solid m.p. 78 - 79°C. and Mannich (60), as 83.5 - 84.5°C.

(c) Preparation of 4-(p-hydroxyphenyl)-butan-2-ol (III)

1.0 g of II (from (b)) was dissolved in 20 ml of methanol to which was added 0.40 g of sodium borohydride in methanol solution. The reaction mixture was kept for an hour and then it was made acidic with dilute sulphuric acid and extracted with ether. The ether extract was washed once with water, dried over anhydrous magnesium sulphate and the dried solution was evaporated under reduced pressure to give 0.80 g of a colourless thick viscous liquid, which did not crystallise from solvents. On keeping the viscous liquid at 0°C. for a day it solidified to give needle crystals. m.p. 67 - 68°C. (Reported 70 - 71°C. (59), 69 - 70°C. (60), 68 - 70°C. (61), 81°C. (62). TLC R_f 0.38.

λ_{max} and λ_{max} (acid). 2033 Å ($\log \epsilon = 3.74$), 2247 Å ($\log \epsilon = 3.87$), 2800 Å ($\log \epsilon = 3.77$).

λ_{max} (base) 2118 Å ($\log \epsilon = 3.82$) 2427 Å ($\log \epsilon = 3.89$) 2966 Å ($\log \epsilon = 3.79$).

ν_{max} (nujol) 3350 $cm.^{-1}$ (OH) 1600 $cm.^{-1}$ 1590 $cm.^{-1}$ and 1500 $cm.^{-1}$ (-C = C - aromatic), 824 $cm.^{-1}$ (para. disubstitution).

ν_{max} (KBr) 3350 $cm.^{-1}$, 1610 $cm.^{-1}$ 1590 $cm.^{-1}$, 1510 $cm.^{-1}$, 850 $cm.^{-1}$, 835 $cm.^{-1}$ 820 $cm.^{-1}$ and 800 $cm.^{-1}$.

N.M.R. spectrum 3.15 τ (4 protons) 4.4 τ (1 proton) 6.19 τ (1 proton) 7.37 τ (2 protons) 8.27 τ (2 protons) 8.78 τ (3 protons).

Synthesis of (III)

0.05 g of (I) was dissolved in 10 ml of 95% ethanol, 2.0 g of 2% sodium amalgam added and the whole

shaken for twelve hours. The solution was then filtered free from the mercury and evaporated to 3 ml. This was added to 10 ml 1N hydrochloric acid and the acid solution extracted with chloroform. The washed chloroform extract on evaporation 'in vacuo' gave 0.04 g of an oily residue. TLC showed this to be a mixture, R_f 0.38 and 0.43. The residue was dissolved in 2 ml of methanol and 50 mg of sodium borohydride added. After fifteen minutes 10 ml dilute hydrochloric acid was added and the resultant solution extracted with chloroform. The residue (0.035 g) obtained after evaporation of the water washed chloroform extract was a clear viscous oil. TLC showed to have an R_f 0.38 and it was identical in all respects to III obtained in (c).

Acetylation of III

0.08 g 4-(p-hydroxyphenyl)-butan-2-ol, 3 ml of pyridine and 6 ml of acetic anhydride were mixed and heated on a water bath for three hours, after which the mixture was evaporated under reduced pressure to remove as much as possible of the excess pyridine and acetic anhydride. Water was added and the resultant mixture extracted with chloroform. The chloroform extract was washed first with dilute sulphuric acid, then with sodium bicarbonate solution, finally with water and evaporated under vacuum to give 0.082 g of a colourless thick viscous liquid. R_f value 0.75.

λ_{max} and λ_{max} (acid) 2101 Å ($\log \epsilon = 3.62$), 2162 Å ($\log \epsilon = 3.69$), 2645 Å ($\log \epsilon = 2.45$) 2703 Å ($\log \epsilon = 2.36$).

On a rapid scan when base was added to the solution of A there was no shift in λ_{max} but a rapid change took place to give the following

λ_{max} (base): 2100 Å ($\log\epsilon$ = 3.5) 2430 Å ($\log\epsilon$ = 3.72) 2959 Å ($\log\epsilon$ = 3.97).

ν_{max}: (film). 1765 $cm.^{-1}$ 1735 $cm.^{-1}$ 1510 $cm.^{-1}$.

N.M.R. spectrum 2.9 τ (4 aromatic protons) 5.4 τ (1 proton) 7.3 τ (2 protons) 8.2 τ (2 protons) 7.75 τ (3 protons) 7.95 τ (3 protons) 8.77 τ (3 protons).

Synthesis of 4-(o-hydroxyphenyl)-butan-2-ol

0.21 g of 4-(o-hydroxyphenyl)-3-buten-2-one was dissolved in 20 ml of 95% ethanol and the resultant solution shaken for twelve hours with 8.0 g of 2% sodium amalgam. The cloudy solution was filtered from the mercury globules. To the filtered solution was added 0.15 g of sodium borohydride and the mixture left for two hours. Excess dilute hydrochloric acid was added and the solution extracted with chloroform. The chloroform extract after a water wash was evaporated under reduced pressure to give 0.163 g of a colourless viscous oil. The oil could not be crystallised.

ν_{max} (film) 3275 $cm.^{-1}$ (OH) 1600 $cm.^{-1}$ 1505 $cm.^{-1}$ (C = C aromatic) 755 $cm.^{-1}$ (ortho disubstituted).

The infrared spectra of compound A and 4-(p-hydroxyphenyl)-butan-2-ol were superimposable. The spectrum of 4-(o-hydroxyphenyl)-butan-2-ol was completely different to that of A.

CHEMICAL EXAMINATION OF COMPOUND D.

Compound D was obtained from the steam distillation procedure of E.3. In general D always contained traces of C, but from one steam distillation procedure a >99% pure D was obtained. It was a viscous liquid and did not crystallise. The R_f 0.66. I.R. spectrum showed the following absorption peaks in the carbonyl region 1779 $cm.^{-1}$, 1732 $cm.^{-1}$.

λ_{max} and λ_{max} (acid). 2041 Å ($\log \epsilon$ = 4.68) 2410 Å ($\log \epsilon$ = 4.69) 2740 Å ($\log \epsilon$ = 3.74).

λ_{max} (base): 2051 Å ($\log \epsilon$ = 4.69) 2420 Å ($\log \epsilon$ = 4.69) 2740 Å ($\log \epsilon$ = 3.75) 3030 Å ($\log \epsilon$ = 3.31).

Hydrolysis of the sample recovered from Column VI.

0.120 g of the substance recovered from the column VI, eluate fraction 5, was treated with 10 ml of 0.1 N aqueous sodium hydroxide and the turbid solution heated on a water bath for fifteen minutes; when it was acidified with 2 N sulphuric acid and the aqueous solution extracted with ether. The residue obtained after evaporation of the ether extract did not crystallise. It was then transferred to a sublimation tube and sublimed at 50°C. to give two zones, one a crystalline substance, the other a thick viscous liquid. The crystalline material had m.p. 120.5° - 121°C. The infrared spectrum of this material was identical in all respects to benzoic acid. A mixed melting point with benzoic acid showed no depression. The viscous liquid was identified as compound A both by infrared spectrum and TLC.

Preparation of the monobenzoyl derivative of 4-(p-hydroxyphenyl)-butan-2-ol:-

0.10 g of 4-(p-hydroxyphenyl)-butan-2-ol was dissolved in 20 ml of 10% sodium hydroxide solution. To this was added with continual shaking benzoyl chloride, until after thirty minutes, a white precipitate was formed. This precipitate was then extracted with chloroform. The chloroform extract was washed with dilute sulphuric acid and then with a dilute solution of sodium bicarbonate and water until the washings were neutral. The washed chloroform extract was dried and then evaporated to dryness to obtain 0.14 g of product. The dry residue was crystallised from benzene/n-hexane mixture to obtain 0.13 g of a white crystalline product m.p. 68 - 69°C. Reported 59 - 60°C. (59), 68 - 69°C. (63). The TLC indicated that it was a single component R_f 0.58. The infra-red spectrum in nujol showed the following absorption bands, 3320 $cm.^{-1}$ (-OH), 1732 $cm.^{-1}$ (O-benzoyl aryl ester)

λ_{max} and λ_{max} (acid): 2033 Å ($\log \epsilon$ = 3.25) 2315 Å ($\log \epsilon$ = 3.22).

λ_{max} (base): 2096 Å ($\log \epsilon$ = 3.38) 2315 Å ($\log \epsilon$ = 3.21).

The R_f corresponded to compound C of extract E.3.

Preparation of the dibenzoyl derivative of 4-(p-hydroxyphenyl)-butan-2-ol

0.10 g of the monobenzoyl derivative was dissolved in 15 ml of pyridine and 0.5 ml of benzoyl chloride and the mixture was heated on a water bath for

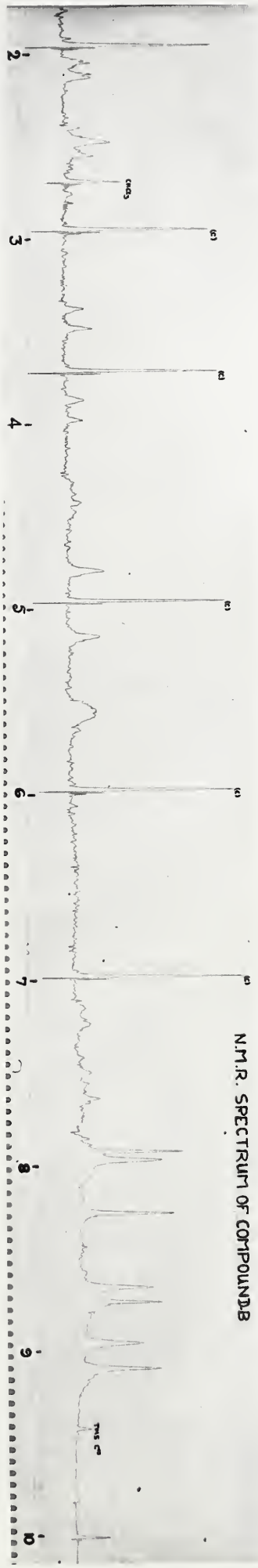
two hours. It was then poured into cold water and vigorously stirred for 30 minutes to destroy excess benzoyl chloride. The mixture was extracted with chloroform. The chloroform extract was washed with dilute sulphuric acid to remove excess pyridine and then several times with dilute solution of sodium bicarbonate to remove excess benzoic acid, and finally with water. The washed extract was dried and then evaporated under reduced pressure to remove the solvent and to obtain 0.12 g of a colourless viscous oil. This could not be crystallised and had R_f 0.66. The product was TLC pure.

ν_{max} (film) 1779 cm.$^{-1}$ (O-benzoyl alkyl) 1732 cm.$^{-1}$ (O-benzoyl aryl)

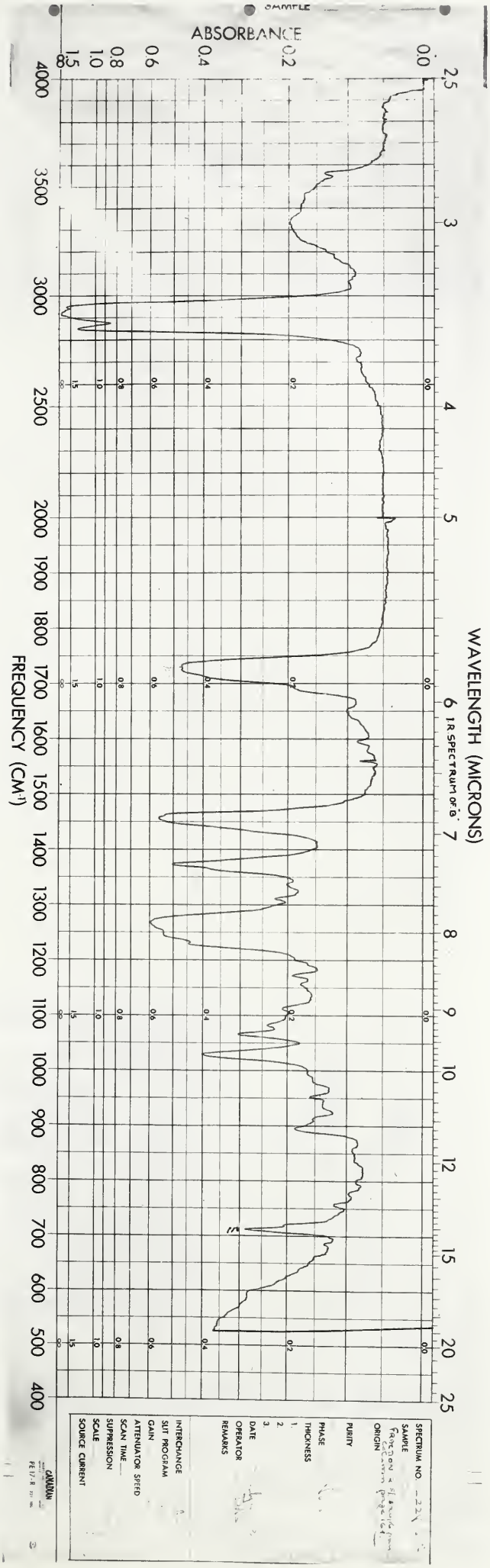
λ_{max} and λ_{max} (acid). 2041 Å ($\log \epsilon$ = 4.68) 2410 Å ($\log \epsilon$ = 4.69) 2740 Å ($\log \epsilon$ = 3.74).

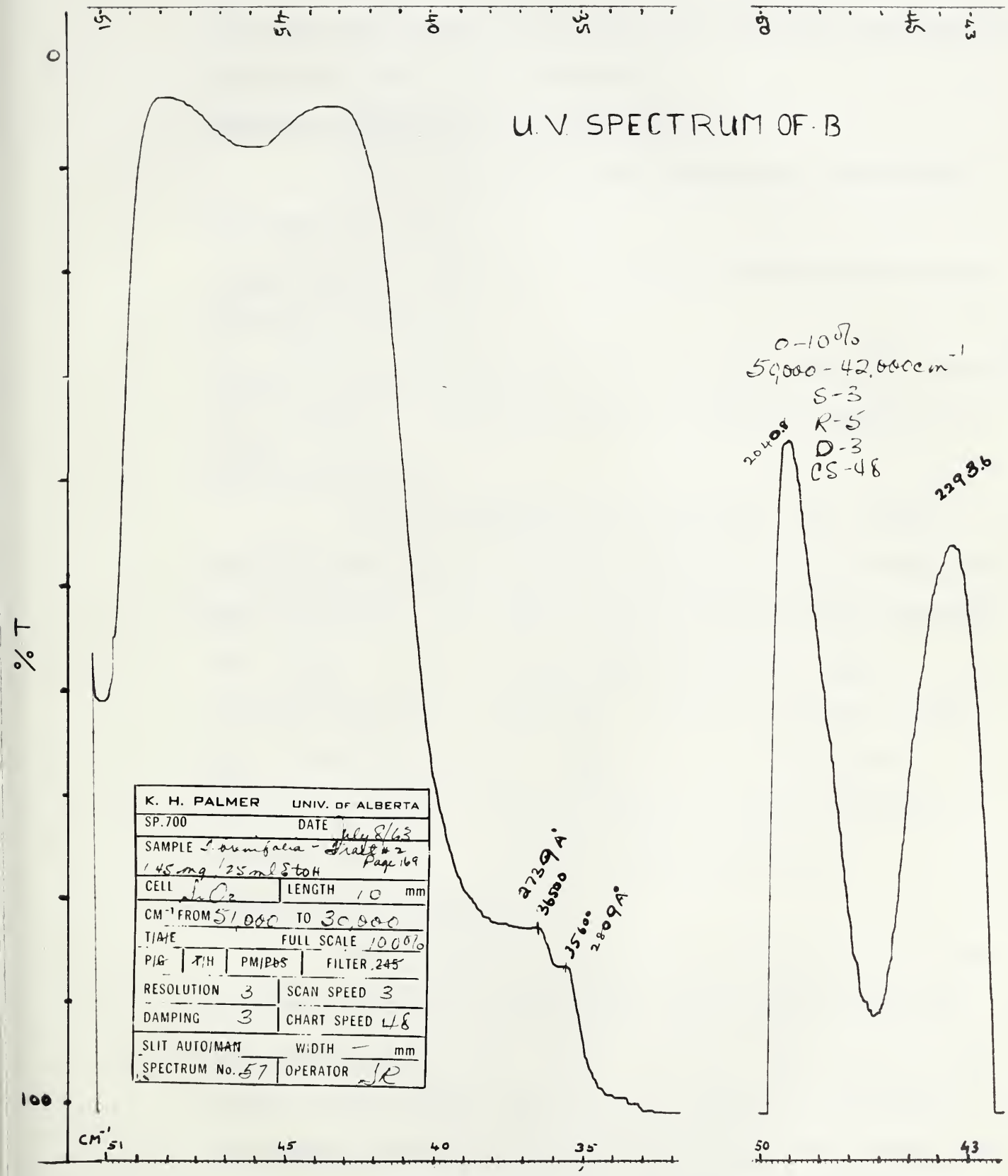
λ_{max} (base) 2054 Å ($\log \epsilon$ = 4.69) 2740 Å ($\log \epsilon$ = 3.75) 3030 Å ($\log \epsilon$ = 3.31).

This dibenzoyl compound was identical in all respects to the pure sample of compound D. Therefore D is the dibenzoyl ester of A and by inference C is identical to 4-(p-benzoyloxyphenyl)-butan-2-ol since it was produced from D by hydrolysis. In columns similar to column VI late eluate fractions were obtained containing only A and C. The I.R. spectrum of these showed a carbonyl absorption at 1732 cm.$^{-1}$ also shown by the mono (aryl) benzoyl compound. No other carbonyl absorption was present. Further using the synthetic


2
3
4
5
6
7
8
9
10
CHCl3
TMS
N.M.R. SPECTRUM OF COMPOUND B

I.R. SPECTRUM of B




U.V. SPECTRUM OF B
% T
0
100
CM⁻¹
51
45
40
35
50
43
0-100%
50,000 - 42,000 cm⁻¹
S-3
R-5
D-3
CS-48
2040 Å
2293 Å
2739 Å
3650
3560
2809 Å
K. H. PALMER UNIV. OF ALBERTA
SP.700 DATE July 8/63
SAMPLE L. ornifolia - Trial #2 Page 169
145 mg 125 ml EtOH
CELL Sa O2 LENGTH 10 mm
CM⁻¹ FROM 51,000 TO 30,000
T/A/E FULL SCALE 100%
P/G T/H PM/PbS FILTER 245
RESOLUTION 3 SCAN SPEED 3
DAMPING 3 CHART SPEED 48
SLIT AUTO/MAN WIDTH — mm
SPECTRUM No. 57 OPERATOR JR

monobenzoyl derivative as seed crystals compound C was crystallised (10 mg) from one of the eluate fractions. This was also found to be identical with the monobenzoyl derivative of A in all respects.

CHEMICAL EXAMINATION OF COMPOUND B:-

This was a white non-crystalline substance TLC pure, R_f 0.41.

ν_{max} (nujol): 3340 $cm.^{-1}$ (-OH intermolecularly bonded) 1730 $cm.^{-1}$ (ester).

λ_{max} and λ_{max} (acid) 2041 Å, 2294 Å, 2739 Å, 2809 Å.

λ_{max} (base): 2132 Å, 2294 Å, 2739 Å, 2809 Å.

N.M.R. spectrum:

9.09 τ (single peak 3 protons $\rightarrow C - C\underline{H}_3$) 8.94 τ (single peak 3 protons $\rightarrow C - C\underline{H}_3$) 8.72 τ (single peak 3 protons $\rightarrow C - C\underline{H}_3$) 8.63 τ (single peak 3 protons $\rightarrow C - C\underline{H}_3$) 8.23 τ (single peak 2 protons - $C\underline{H}_2$ - 7.96 τ (3 protons - O.CO $C\underline{H}_3$) 7.92 τ (3 protons - OCO $C\underline{H}_3$) 5.58 τ (2 protons - $C\underline{H}_2$ -) 5.19 τ (1 proton) 4.63 τ (1 proton) 4.47 τ (1 proton) 4.0 τ (1 proton) 3.43 τ (1 proton) 2.38 τ (4 aromatic protons).

Permanganate oxidation

The sample chosen for this reaction was from a column similar to column II: the eluate fractions corresponding to (131 - 140). By TLC the material was B and A with larger amounts of A than B. Although B was present only as the minor component, the sample was used without purification for the oxidation reaction, since the structure of A was known. 160 mg. of this

sample were treated with 20 ml of 2N aqueous sulphuric acid solution and finely powdered potassium permanganate was added, a little at a time. The reaction was carried out at about 90°C. (on a boiling hot water bath). The addition of the permanganate was continued until a pink colour persisted. The mixture was then heated for another two hours on the water bath. After cooling the excess permanganate and the precipitated manganese dioxide were decomposed with solid sodium meta-bisulphite, the reaction mixture was then extracted with ether in a continuous extractor for twenty-four hours. The ether extract was washed, dried and the dried ether solution was evaporated to dryness under vacuum to give 25 mg of residue. This product did not crystallise. On sublimation at 50°C. at about 0.05 mm/Hg, 1.8 mg of a white crystalline sublimate were obtained. This had m.p. 130 - 131°C. Infra-red spectrum of this sample was identical in all respects with phthalic anhydride. Mixed melting point with phthalic anhydride showed no depression. TLC on silica gel plate using 95% ethanol/ water/25% aqueous ammonia (25:3:4) solvent mixture gave single spot R_f 0.27, identical to phthalic anhydride.

PAPER CHROMATOGRAPHY OF BIFLAVANOIDAL COMPONENTS OF *T. brevifolia*:-

The alkaline mother liquor from the extraction of *T. brevifolia* by method (a) (section (ii) were brought to pH 3 by the addition of concentrated hydrochloric acid. The resultant golden yellow aqueous

solution was extracted with n-butanol until the butanol extract was colourless. The combined butanol extracts were washed with water. Much of the water in the butanol extract was removed by anhydrous sodium sulphate and the resultant solution evaporated to dryness 'in vacuo'. The resultant brown tarry mass was extracted with anhydrous methanol and the methanolic extract evaporated to dryness.

Paper chromatography using the conditions of Di Modica, Rossi and Rivero (58) on the methanol extract, the heptane extract of the plant material and also E.3 failed to reveal any traces of biflavanoidal pigments. This was in contrast to a similar extract from T. baccata when spots having a flavanoidal reaction corresponding to the biflavanoidal components reported by the above authors, were easily detected.

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